Genotypic and phenotypic characterisation of *Streptococcus uberis*

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Abstract

Streptococcus uberis is an important bovine mastitis pathogen, which places a significant financial burden upon the dairy industry. Determining the genetic diversity of a collection of field isolates and the mechanisms by which *S. uberis* colonises the host were the general aims of this project, in particular the determination of the basis for bacterial persistence despite antibacterial therapy. Multi-locus sequence typing identified high levels of recombination within the population, but also a single dominant clonal complex which comprised nearly all sequence types which were isolated from more than one animal. The dominant clonal complex also comprised isolates, derived, however, from both persistent and non-persistent infections, but RAPD typing demonstrated that these isolates can differ in genetic composition elsewhere in the genome. Whole genome sequencing of additional *S. uberis* isolates confirmed that despite significant homology between much of these genomes, novel genetic material was commonly obtained by phage insertion and horizontal gene transfer. Isolates with identical housekeeping sequences are thus highly likely to differ in their virulence gene repertoires. In this study, the potential for differentiating *S. uberis* isolates based instead upon protein profiles derived from mass spectrometry of disrupted whole cells was therefore also explored. Differentiation between small numbers of isolates was achieved after optimisation of this protocol, however, discriminatory ability and reproducibility were somewhat compromised when the technique was scaled up to analyse 50 Italian isolates. During the period of study, profile differences between persistent and non-persistent isolates could not be explored.

Basic methods were thus also utilised in an attempt to identify factors which promoted bacterial survival *in vitro*; and a defined medium, representative of the udder environment, was optimised for this purpose. The use of this medium permitted the demonstration that *S. uberis* was reliant upon magnesium and manganese for proliferation and that, interestingly, the absence of iron did not inhibit bacterial growth. It was also shown that *S. uberis* had the ability to directly utilise casein, identifying a potential alternative pathway for the acquisition of essential nutrients from nutritionally-limited environments. It was also observed that to a limited extent *S. uberis* seemed to produce a siderophore. Although this remains to be confirmed, it may correlate with the observation that iron, although not essential for proliferation, improved the growth rate of the bacterium. It was also notable that most novel genes, identified from *S. uberis* genome sequences, exhibited functions for nutrient metabolism, demonstrating that flexibility in nutrient acquisition is central to the ability of the bacteria to adapt, permitting survival in vastly different environments. The use of the defined medium also demonstrated that *S. uberis* was able to form

biofilms; this ability being variable depending on the growth conditions used and the isolate studied. Most significantly, under conditions representative of the mammary gland, there was an apparent trend for high levels of biofilm formation to correlate with isolates from persistent infections. Biofilm formation by *Staphylococcus aureus* is considered to be pivotal to the development of chronic mastitis, thus, biofilms may similarly play a role in *S. uberis* persistence.

In an attempt to identify the molecular basis for *S. uberis* biofilm formation, genes with homology to those of the intercellular adhesion (*ica*) operon, well described for their involvement in *Staphylococcus epidermidis* and *S. aureus* biofilm formation, were identified in the genome sequence of *S. uberis* 0140J. A targeted mutagenesis protocol was optimised to 'knock out' these genes and observe the subsequent effects of these mutations on biofilm formation. During the course of this study, two of these potential biofilm genes (*hasA* and SUB 0809) were deleted from the *S. uberis* 0140J chromosome. Surprisingly, deletion of these genes did not retard subsequent biofilm formation, but instead biofilm formation was dramatically improved in the mutant strains.

Characterisation of mastitis-causing *S. uberis* strains and a detailed understanding of the pathogenicity of the organism are required to further the development of a successful vaccine. The research presented in this thesis has increased the knowledge of these important research objectives and optimised techniques which will allow further advancement of knowledge in this field.

Declaration

The work reported in this thesis was carried out under the supervision of Dr Michael C. Fontaine and Professor David G. E. Smith at the Moredun Research Institute and the Faculty of Veterinary Medicine, University of Glasgow. All results presented, unless otherwise stated, are the sole work of this author, as is the composition of this thesis.

Signed: Date:

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Abbreviations

Acc. # or Acc. No.	NCBI Accession number
agr	Accessory Gene Regulator
AIP	Auto-inducing peptide
Approx.	Approximately
arcC	Carbamate kinase gene
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
B	Bovine
BA	Blood agar
Bap	Biofilm Associated Protein
BCC	BURST/eBURST assigned Clonal Complex
BHI	Brain Heart Infusion
BL	Back-left udder quarter
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BR	Back-right udder quarter
BSA	Bovine Serum Albumin
ca.	*circa* (around)
$CaCl^2$	Calcium chloride
CAMP factor	Co-hemolysin (discovered by Christie, Atkins and Munch-Petersen)
CAS	Chrome azurol S
CC	Clonal Complex
CDM	Chemically-defined Medium
CDMch	CDM containing 0.5 % hydrolysed casein
CDMcas	CDM containing 0.5 % casein
CDS	Coding sequence
cDNA	Complementary deoxyribonucleic acid
c.f.u/ml	Colony forming units per ml
ch	Casein hydrolysate/hydrolysed casein
cm	Centimetre
Cm^r	Chloramphenicol resistance
CRA	Congo Red Agar
Da	Dalton (Atomic mass unit)
DCC	Database assigned Clonal Complex
ddl	D-alanine-D-alanine ligase gene
dH_20	Water
DI	Discriminatory index
DLV	Double Locus Variant
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNaSP	DNA Sequence Polymorphism
dN/dS	Ratio of the number of non-synonymous over synonymous substitutions
dNTP	Deoxyribonucleotide triphosphate
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
eBURST	Based Upon Related Sequence Types
ECM	Extracellular matrix
ECMP	Extracellular matrix proteins
EDDA	Ethylenediamine-N,N'-diacetic acid
EDTA	Ethylenediaminetetraacetic acid
e.g.	*exempli gratia* (for example)
Em^r	Erythromycin resistance
Fe	Iron

FL	Front-left udder quarter
FPP	Five Point Plan for mastitis control
FR	Front-right udder quarter
FR	Frosinone
g	Gram
GAG	Glycosaminoglycans
GapC	Glyceraldehyde-3-phosphate dehydrogenase
gDNA	Genomic deoxyribonucleic acid
gki	Glucose kinase gene
HasA	Hyaluronate synthase
HasB	UDP-glucose dehydrogenase
HasC	UDP-glucose pyrophosphorylase
HCl	Hydrochloric acid
HDTMA	Hexadecyltrimethyl ammonium bromide
h	Hour(s)
Hz	Hertz (SI unit of frequency)
Ica	Intercellular adhesion protein
ICM-MS	Intact Cell MALDI-ToF MS
ICMS	Intact Cell Mass Spectrometry
ID	Identity/Identifier
i.e.	*id est* (that is)
IgG	Immunoglobulin G
IgM	Immunoglobulin M
I^{S}_{A}	Standardised index of association
JPEG	Joint Photographic Experts Group
kb	Kilo base
kDa	Kilo Dalton
KPa	kilo Pascal
kV	Kilo volt
l	Litre
LAB	Lactic Acid Bacteria
LD	Linkage Disequilibrium
LB	Luria-Bertani Broth
Lbp	Lactoferrin-binding protein
LT	Latina
Ltd	Private Limited Company
LuxS	S-ribosylhomocysteinase gene
M	Molar
m	Minute(s)
mA	Milliamp
MAC-T	Bovine mammary epithelial cell line
MALDI-ToF	Matrix assisted Laser Desorption Ionisation Time of Flight
mg	Milligram
Mg	Magnesium
MIC	Minimum Inhibitory Concentration
ml	Millilitre
MLST	Multi-Locus Sequence Typing
mM	Millimolar
Mn	Manganese
mRNA	Messenger ribonucleic acid
MRSA	Methicillin Resistant *Staphylococcus aureus*
ms	Microseconds
MS	Mass Spectrometry
MSP	Main Spectral Projection

MSSA	Methicillin Sensitive *Staphylococcus aureus*
MtuA	Metal transporter uberis A
m/z	Mass to charge ratio
n	Number
NaOH	Sodium Hydroxide
NADH	Reduced Nicotinamide Adenine Dinucleotide
NCBI	National Centre for Biotechnology Information
ND	Not done
ng	Nanogram
nm	Nanometre
NP	Non-persistent
NTA	Nitrilotriacetic acid
^{o}C	Temperature in degrees centigrade
O	Ovine
OD	Optical density
Opp	Oligopeptide transport protein
ORF	Open Reading Frame
P	Persistent
PAGE	Polyacrylamide gel electrophoresis
PauA/B	Plasminogen activator uberis A/B
PBS	Phosphate buffered saline
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PFGE	Pulsed-Field Gel Electrophoresis
PIA	Polysaccharide Intercellular Adhesin
PMN	Polymorphonuclear neutrophils
pmol	Picomole
PNAG	Poly-N-acetyl-β-1,6-glucosamine
PrtP	Extracellular serine protease
psi	Pounds per square inch
QC	Quantum Clustering
QS	Quorum Sensing
RAPD	Random Amplified Polymorphic DNA
rATP	Recombinant Adenosine triphosphate
recP	Transketolase 2 gene
REF	Restriction Endonuclease Fingerprinting
REP	Repetitive Extragenic Palindromic Typing
RI	Rieti
RNA	Ribonucleic acid
RNase	Ribonuclease
RM	Roma
rpm	Revolutions per minute
RPMI	Media developed at Roswell Park Memorial Institute
rRNA	Ribosomal RNA
RT	Room temperature
RT	Reverse transcription
RT-PCR	Reverse transcription PCR
s	Second(s)
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SIBIs	Species identifying biomarker ions
SLV	Single Locus Variant
SNP	Single Nucleotide Polymorphism

SOC	Super Optimal Broth
SP	Slime Producing
S. ub	*Streptococcus uberis*
SCC	Somatic Cell Count
ST	Sequence Type
START	Sequence Type Analysis and Recombinational Tests
SUAM	*S. uberis* adhesion molecule
SUPA	*S. uberis* plasminogen activator
TBS	Tris-Buffered Saline
tdk	Thymidine kinase gene
TFA	Tri-fluoroacetic acid
TIFF	Tagged Image File Format
tklA	acetyl-coA acetyltransferase gene
TLV	Triple Locus Variant
tpi	Triosephosphate isomerase gene
Tris-HCl	Tris-Hydrochloride
U	Units
UDP	Uridine diphosphate
UK	United Kingdom
US	United States
UV	Ultraviolet
UV-Vis	Ultraviolet-visible
V	Volt
VT	Viterbo
W/T	Wild-type
yqiL	acetyl-coA acetyltransferase 2 gene
μF	Microfarad (SI unit capacitance)
μg	Microgram
μl	Microlitre
μm	Micrometre
μM	Micromolar
Ω	Ohm (SI unit of electrical resistance)
Δ	Delta (for gene deletion)
× *g*	Times gravity

Chapter 1: General Introduction

1.1 *Streptococcus uberis* History and Taxonomy

The genus *Streptococcus* belongs to the larger group of microaerophilic, Gram-positive microorganisms known as the lactic acid bacteria (LAB), which ferment hexose sugars through homo or hetero fermentative pathways to produce lactic acid. These bacteria are indigenous to food-related habitats such as plant and milk environments and are frequently associated with mucosal surfaces in animals. In this capacity, *Streptococcus uberis* is no exception, and is frequently isolated from cattle with mastitis, whilst also being ubiquitous in the dairy environment (Cullen, 1966; Cullen and Little, 1969). Taken as a whole, the streptococci encompass a diverse range of both pathogenic and commensal species, inhabiting a vast host range which includes humans, farmed and domestic animals. Streptococci are non-motile, non-sporing, catalase-negative, facultatively-anaerobic chemo-organotrophs, requiring nutritionally rich media for growth. Cells are spherical or ovoid, 0.5 to 2.0 μm in diameter, and exist in pairs or chains of varying length.

S. uberis, originally designated Group III streptococci, can be differentiated from other mastitis causing streptococci by a lack of β–hemolysis on blood agar, hydrolysis of esculin and sodium hippurate, and fermentation of inulin, lactose, trehalose, mannitol, ribose, sorbitol and salicin (Facklam, 1977; Holt *et al.*, 1994; McDonald and McDonald, 1976). These tests can now be conducted routinely using commercial Strep-Zym (Lammler, 1991) or API test systems (Poutrel and Ryniewicz, 1984). Group III streptococci were renamed *S. uberis* in 1932 (Diernhofer, 1932), and since then many attempts have been made to serologically profile this species. Classification of most streptococcal species is based largely upon serology of cell wall carbohydrates, using Lancefield's classification system (Lancefield, 1933). Simple identification of *S. uberis* using this method is however, not possible, as no group antigens are conserved among all *S. uberis* strains. A cell wall antigen which bound to immunised rabbit serum was, in one report, found to be specific for all *S. uberis* strains tested; however, the identity of this protein was not discovered (Jones and Norcross, 1983). In the literature *S. uberis* has previously been classified as a member of the viridians (Facklam, 1977), pyogenic (Hardie and Whiley, 1997) or parapyogenic (Bridge and Sneath, 1983) streptococcal groups. Clearly *S. uberis* is an unusual *Streptococcus* species which fails to be satisfactorily classified by conventional methods. Similarities between *S. uberis* and *Streptococcus pyogenes* ecology prompted the suggestion, however, that similar hygiene practices used to control human *S. pyogenes* infections could be beneficial for preventing the spread of bovine *S. uberis* (Sweeney, 1964).

In 1979, two distinct *S. uberis* genotypes (Types I and II) were determined, based upon DNA hybridisation studies (Garvie and Bramley, 1979). Not until 1990 did comparison of 16S ribosomal RNA sequences of both genotypes confirm that despite morphological and serological homology, types I and II were phylogenetically distinct, prompting the authors to propose type II be re-designated as a new species, *Streptococcus parauberis* (Williams and Collins, 1990). Genetic differentiation of *S. uberis* and *S. parauberis* was further demonstrated using restriction fragment length polymorphism techniques (Jayarao *et al.*, 1991), PCR (Hassan *et al.*, 2001) and sequencing of specific gene fragments (Alber *et al.*, 2004). Furthermore, sequencing of chaperonin and heat shock genes later confirmed that *S. uberis* is indeed most similar to pyogenic streptococci, particularly *Streptococcus porcinus* (Alber *et al.*, 2004; Kawata *et al.*, 2004).

Molecular techniques permitting differentiation of *S. uberis* from *S. parauberis*, subsequently allowed further demonstration that *S. uberis* predominates in the bovine mammary gland, being a frequent cause of mastitis, whilst *S. parauberis* was very infrequently associated with infection in cattle (Alber *et al.*, 2004; Hassan *et al.*, 2001; Jayarao *et al.*, 1991; Pitkala *et al.*, 2008). *S. parauberis*, along with a diverse group of additional LAB, has since been linked to disease in fish (Michel *et al.*, 2007). Characterisation of *S. uberis* using molecular techniques is further discussed in **Chapter 3**.

In 2001, the first efforts to sequence an *S. uberis* genome commenced. The chosen strain was 0140J (ATCC BAA-854), a bovine mastitis isolate for which a body of work exists in the published literature. This strain was isolated in 1973 from a case of clinical mastitis, and was subsequently shown to be pathogenic in lactating animals (Coffey *et al.*, 2006; Hill, 1988a). The 0140J genome sequence was eventually published in 2008, comprising a single chromosome of 1,852,352 bp with a G+C content of 36.63 % (Ward *et al.*, 2009). Approximately 11 % of predicted coding sequences were specific to *S. uberis* and approx. 40 % were homologous to all previously sequenced streptococci; the most orthologous sequences were shared with pyogenic streptococci, confirming earlier observations (Alber *et al.*, 2004; Kawata *et al.*, 2004; Ward *et al.*, 2009). Specifically, the chromosome structure was closest to that of *Streptococcus equi* subsp. *zooepidemicus* and *S. pyogenes* (Ward *et al.*, 2009). Analysis of the *S. uberis* genome sequence revealed a wide diversity of genes encoding proteins associated with carbohydrate utilisation and energy metabolism. This infers an ability to adapt and survive within challenging environments, and serves to highlight the opportunistic

nature of this pathogen (Ward *et al.*, 2009). Significantly, the genome sequence also presents an invaluable resource for future genomic and proteomic studies.

Although references to *S. uberis* are predominantly concerned with bovine mastitis, the organism is also associated with mastitis in sheep (Hariharan *et al.*, 2004; Mork *et al.*, 2007), and infrequently in buffalo (Jaffery and Rizvi, 1975; Moroni *et al.*, 2006), goats (Al-Graibawi *et al.*, 1986) and pigs (Sumner, 1957). *S. uberis* has only on rare occasions been linked to infections in humans and even these cases are disputed (Facklam, 2002), as typing demonstrated that suspected human strains were so divergent from bovine strains that they were unlikely to be *S. uberis* (Williams and Collins, 1991). The organism is, however, commonly isolated from the periodontal site of healthy humans, where it is believed to be commensal, producing hydrogen peroxide which inhibits growth of periodontal pathogens (Hillman *et al.*, 1985). As such, a clinical trial of a probiotic mouthwash containing *S. uberis* is currently ongoing (Zahradnik *et al.*, 2009).

1.2 Mastitis

Mastitis is broadly defined as inflammation of the mammary gland. Inflammation can occur as a result of physical damage, although, it is more commonly the manifestation of a mammary gland infection. In cattle, when bacteria invade the udder, the normal host immune response causes the release of inflammatory mediators, leading to a massive influx of leukocytes, mainly polymorphonuclear neutrophils (PMN), into the mammary gland (Craven and Williams, 1985). This cascade is responsible for the resulting inflammation. Dependent upon the invading pathogen, subsequent bacterial multiplication within the gland, toxin release and increased movement of host factors leads to membrane, cell and tissue damage. Severity of mastitis is thus dependent upon both the organism involved and the intensity of the host immune response (Hill, 1981). Inflammation reduces the number and activity of milk producing epithelial cells, resulting in reduced milk production. Milk quality is also affected by compositional changes arising from increased blood components such as albumin entering the milk, and reduction in normal constituents such as fat (Hogarth *et al.*, 2004). Decreased milk production and quality account for by far the greatest financial losses caused by mastitis, with costs of veterinary bills and replacement of culled animals also significant (Hillerton *et al.*, 2005). Mastitis therefore represents the single greatest cost to the dairy industry, with over 1 million cases occurring annually in the UK.

Mastitis-causing infections are defined as either clinical or sub-clinical, dependent upon the presence or absence of physical symptoms. Clinical mastitis is readily diagnosed by visible abnormalities in the udder and/or the milk. Clinical symptoms in cattle range from mild swelling to extreme cases of dehydration, loss of appetite and even death due to gangrene development (Jain, 1979; Stableforth, 1950); however, mastitis is often not serious. Chronic infections may develop however, requiring the animal to be culled. In sheep, symptoms are similar and infections often cause irreversible damage to the gland such that animals cannot feed lambs properly, as treatment in sheep is not economically viable animals are thus commonly culled (Mork *et al.*, 2007; Watson and Buswell, 1984). As bacterial culturing is not conducted routinely, sub-clinical mastitis is usually only diagnosed following observations of significantly increased somatic cell count (SCC; number of leukocytes per ml of milk), or from reduced milk production or quality. A high SCC arises as a direct result of the influx of immune cells into the mammary gland in response to an invading pathogen, but which has not at that point resulted in an obvious inflammatory response. Diagnoses of sub-clinical mastitis are normally made using the California Mastitis Test (Leach *et al.*, 2008), whilst bulk tank milk observations monitor udder health of the herd in general, being a reflection of farm hygiene levels (Jayarao *et al.*, 2004). Acceptable levels for bulk tank SCC are set by regulatory bodies, such that a low SCC indicates low levels of mastitis within the herd. As the bulk SCC partially dictates the price or premium received for the milk, there is a strong incentive for farmers to reduce the frequency and severity of mastitis infections within the herd.

Numerous microorganisms are known to cause bovine mastitis, with bacteria accounting for more than 90 % of infections (Watts, 1988). Mastitis causing bacteria are generally regarded to be either contagious or environmental in nature, depending upon the route by which they most commonly cause infection. *Staphylococcus aureus* and *Streptococcus agalactiae* are well-defined 'contagious' pathogens with infection being spread from cow to cow from the primary reservoir of the infected udder quarter. Contagious transmission within a herd has been particularly well demonstrated using pulsed-field gel electrophoresis (PFGE), where the same strain of *S. agalactiae* was identified from multiple infected animals in the same herd, whilst different strains were observed between herds (Baseggio *et al.*, 1997).

In order to reduce the incidence of mastitis among dairy herds, research conducted at the National Institute for Research in Dairying in the 1950s and 60s led to the development of a "five point plan (FPP)" for mastitis control (Kingwill *et al.*, 1970;

Neave *et al.*, 1969). This control programme was implemented in the UK in the late 1960s following publication of results from field trails demonstrating the effectiveness of the measures, which included post-milking teat dipping and routine antibiotic therapy (Dodd, 1983). The widespread implementation of these measures produced a marked decline in clinical mastitis cases in England and Wales by the late 80s (Booth, 1988). Particularly, *S. agalactiae* was readily eradicated as it is sensitive to antibiotic therapy and unable to survive in the bovine environment outside the mammary gland (Jain, 1979). As such the FPP has been widely adopted in dairying regions worldwide. Mastitis has not however, been prevented, and a 2007 study in England and Wales identified between 40 and 70 clinical cases per 100 cows per year (Bradley *et al.*, 2007). Despite the clear benefits of the FPP, it does not effectively tackle the issue of 'environmental' pathogens such as *Escherichia coli* and *S. uberis* (Smith *et al.*, 1985; Watts, 1988). Even in the 1970s, concerns were raised about the frequent isolation of *S. uberis* and *E. coli* from two herds in which dry cow therapy and teat dipping were routinely conducted (Marr, 1978a; Marr, 1978b). In contrast to contagious pathogens, these bacteria are transmitted to the cow from the environment, such as contaminated pasture and bedding, with the mammary gland being a secondary reservoir of infection. Even the strictest adherence to the good hygiene practices of the FPP thus cannot eliminate the risk of constant re-infection from the environment. The need to identify alternative means of controlling environmental pathogens, including *S. uberis*, is therefore clear.

1.3 *S. uberis* etiology and incidence

S. uberis has been isolated from the lips, belly, rectum, vagina, teat skin (Cullen, 1966), uterus (McDougall, 2005) and rumen of the cow (Cullen and Little, 1969), and has also been isolated from bedding and soil where herds have grazed, and even from water, plant matter and flies from the farm environment (Cullen and Little, 1969; Zadoks *et al.*, 2005b). Despite being ubiquitous on the cow, there are no reports of *S. uberis* being associated with any bovine condition other than mastitis. The udder surface/skin was considered to be the primary reservoir for infection, as bacterial loads were generally higher than in milk and a greater number of isolations were made from this site (Cullen, 1966; Sweeney, 1964). Isolation of *S. uberis* from the lips was also frequent, suggesting that an additional route of transmission by licking should be considered (Cullen, 1966). Contrastingly, the teat skin showed much lower *S. uberis* carriage, questioning whether colonisation of the teat is required as a precursor to intra-mammary infection (Cullen, 1966). From these early observations, the theory that

infection of the mammary gland may be due to secondary infection from other skin sites such as the udder and the belly which serve as constant reservoirs for infections was developed (Cullen, 1966; Cullen and Little, 1969; Sweeney, 1964). Faecal shedding is considered to be an additional route by which *S. uberis* is transmitted to animals, as this organism is frequently isolated from soil samples where animals are housed but is not identified from soil that has not been exposed to cow faeces (Cullen and Little, 1969; Zadoks *et al.*, 2005b). Thus *S. uberis* may also colonise the bovine gastro-intestinal tract.

Research has demonstrated that, unlike many other mastitis pathogens, mastitis caused by *S. uberis* occurs throughout the year and at all stages of lactation, drying-off and calving (Francis *et al.*, 1986; Smith *et al.*, 1985; Todhunter *et al.*, 1995; Todhunter *et al.*, 1985), with the organism equally able to survive in the dry gland (Hill, 1988a). The bovine mammary gland does appear, however, to be more susceptible to new *S. uberis* infections during the peri-partum period, resulting in an increased incidence of mastitis at calving (McDonald and Anderson, 1981). Mastitis-causing infections attributable to *S. uberis* are rarely severe (Deluyker *et al.*, 2005) and in one study 78 % of infections were shown to cure spontaneously (McDougall *et al.*, 2004). Repeat infections are however common and observations of chronic *S. uberis* infections and persistence despite antibiotic therapy are also described (Doane *et al.*, 1987; Milne *et al.*, 2005; Stableforth, 1950). Treatment with many antimicrobials, including pirlimycin hydrochloride, was in fact shown generally to induce no greater resolution of infections with mastitis-causing pathogens than where no therapy was administered (Apparao *et al.*, 2009), with the exception of *S. agalactiae* (Wilson *et al.*, 1999). It is now also widely accepted that susceptibility to antimicrobials *in vitro* is mostly unrelated to the outcome *in vivo* (Apparao *et al.*, 2009; Sinha, 1984) and that a wide range of variables such as animal age, lactation stage, treatment programme and pathogen involved, vastly affect the outcome of antimicrobial treatment (Deluyker *et al.*, 2005; Wilson *et al.*, 1999). Nevertheless, a small proportion of *S. uberis* isolates have been shown *in vitro* to resist certain antibiotics, whilst in general isolates were susceptible (Bengtsson *et al.*, 2009; Milne *et al.*, 2005; Pitkala *et al.*, 2008). Intracellular killing of *S. uberis* in mammary epithelial cells was also achieved *in vitro* with high concentrations of penthamate hydriodide (Almeida *et al.*, 2007), but again, this may not reflect the *in vivo* situation. Significantly, treatment with sub-lethal concentrations (0.3 to 0.5 x minimum inhibitory concentration, MIC) of the broad spectrum antibiotic ciprofloxacin induced greater *S. uberis* mutagenesis, accelerating the development of rifampin resistance (Varhimo *et al.*, 2008), demonstrating a major problem associated with high

levels of antibiotic usage. Spraying teats with an iodine sanitizer also had minimal effect on the subsequent incidence of clinical mastitis caused by *S. uberis* (Lopez-Benavides *et al.*, 2009), whilst teat sealants have shown some promising results (Parker *et al.*, 2007) but require further investigation.

Numerous surveys have attempted to quantify the incidence of bacterial mastitis pathogens in various countries; however, it is difficult to compare these studies due to the wide number of associated variables, including the stage of lactation, herd numbers and bias problems resulting in over-representation of certain species. Incidence clearly varies globally, but in many countries *S. uberis* is a very important mastitis pathogen, featuring prominently in surveys from the UK, US and New Zealand, dating back to the 1960s (Dodd *et al.*, 1969; Francis *et al.*, 1986; Guterbock *et al.*, 1993; Hillerton and Berry, 2005; McDonald and McDonald, 1976). Two UK surveys conducted between 1999 and 2005 demonstrated the significance of *S. uberis* as a leading causative agent of clinical mastitis, being isolated from 37 % (Milne *et al.*, 2002) or 32 % (Bradley *et al.*, 2007) of milk samples respectively. One survey also identified *S. uberis* as the second most commonly isolated pathogen from sub-clinical cases of mastitis (Bradley *et al.*, 2007). In New Zealand, where bacteria were isolated from clinical mastitis, *S. uberis* was identified in 63 % of cases (McDougall *et al.*, 2004). In Finland, *S. uberis* was the third most common cause of clinical and sub-clinical mastitis (Koivula *et al.*, 2007) and in Estonia 19.1 % of isolates from clinical mastitis of freshly calved heifers were found to be *S. uberis* (Kalmus *et al.*, 2006). In contrast, in a Norwegian survey *S. uberis* was isolated from just 1.8 % of clinical mastitis samples (Waage *et al.*, 1999).

Despite routine antibiotic therapy and post milking teat dipping, environmental pathogens like *S. uberis* remain a serious problem for the dairy industry. More effective control measures are clearly required to reduce infections caused by this pathogen. This would positively impact upon the overall mastitis levels and represent a significant financial saving. The route by which *S. uberis* causes infection is still only speculated, despite being extensively researched. As the continued use of therapeutic antibiotics becomes a less-favourable option, the dairy industry now looks to the development of alternative approaches for mastitis control, including the development of effective vaccines. A traditional approach to vaccine design is to identify factors associated with the ability of a given organism to cause disease. These factors then become the basis of a vaccine, in order to target the host's immune response against the pathogen and interfere with the colonisation or persistence of the organism. In order to develop such vaccines, gaining knowledge regarding the pathogenesis of the organism is vital, and in

this respect *S. uberis* is no different. As discussed below, several virulence factors that contribute to the ability of *S. uberis* to evade host defences, adhere to and invade host cells, and survive in the udder environment have been described to-date and many have been considered as vaccine candidates.

1.4 *S. uberis* virulence factors

While the full relationship between *S. uberis* and its host has yet to be elucidated, it is known that the colonisation of, and persistence within the mammary gland by this organism is a multifactorial process. The contribution of a number of different bacterial proteins is required at different stages of infection, these being involved in processes as diverse as nutrient acquisition, evasion of the host immune response and adherence to and invasion of host epithelial cells. Being armed with the correct molecular arsenal allows *S. uberis* to persist within the mammary gland, rendering it highly-refractory to subsequent attempts to eradicate the infection.

1.4.1 Adhesion to host cells

Initial adherence of bacteria to cells of the mammary gland mucosa is important in the early stages of mastitis pathogenesis, acting as a bridge which allows subsequent internalisation of the bacteria into these cells. Direct adhesion of *S. uberis* to host cell surface glycosaminoglycans (GAGs) was demonstrated (Almeida *et al.*, 1999a), however, adhesion was promoted by interaction of *S. uberis* with extracellular matrix proteins (ECMP) particularly collagen (Almeida *et al.*, 1999b) and lactoferrin (Fang *et al.*, 2000). In one study, bacterial capsule promoted adherence of *S. uberis* to ECMP but reduced direct adherence to epithelial cells (Almeida *et al.*, 1996). As with other pathogenic streptococci, increased production of ligands occurred in the presence of ECMP, which in turn enhanced both direct binding to host cells as well as the binding of the bacteria to ECMP which promoted indirect adherence to host cells via a "molecular bridge" (Almeida and Oliver, 2001; Love *et al.*, 1997). Bacterial ligands responsible for molecular bridge formation and cellular adhesion may therefore be both constitutively present in *S. uberis* outer membranes and expressed in response to host stimuli. Different GAGs and milk proteins were studied, and brief pre-treatment in heparin sulfate and β-casein most notably increased subsequent adhesion and internalisation of *S. uberis* (Almeida *et al.*, 2003). Many such adhesins have been characterised in *S. aureus*, and indeed a vaccine comprising four adhesion factors, offered protection to mice against subsequent intra-mammary challenge (Castagliuolo *et al.*, 2006).

Two *S. uberis* proteins which bind lactoferrin, an iron binding glycoprotein found in milk and dry cow secretions, have been described, these being the lactoferrin-binding protein (Lbp), an M-like protein (Moshynskyy *et al.*, 2003), and the *S. uberis* adhesion molecule, SUAM (Almeida *et al.*, 2006). Adherence of *S. uberis* to epithelial cells was however, found not to be reliant upon Lbp (Moshynskyy *et al.*, 2003), but inhibition of adhesion was seen with antibodies against SUAM or bovine lactoferrin (Almeida *et al.*, 2006; Patel *et al.*, 2009). An association between host factors and bacterial cells is important for infection; this interaction between *S. uberis* and mammary cells is clearly complex and likely to involve multiple proteins, which as yet have not been identified. Once attached to the host cell, additional mechanisms are required to further stimulate internalisation into host cells.

1.4.2 Internalisation

Internalisation within host cells offers *S. uberis* a distinct advantage through permitting it respite from the host's immune response as well as from antimicrobial agents. Distinct strains of *S. uberis* expressing different virulence factors, including capsule, have been shown to be capable of invading bovine mammary epithelial (MAC-T) cells, *in vitro*, with differing abilities (Matthews *et al.*, 1994a). Survival of *S. uberis* was seen for up to 120 h in MAC-T cells without obvious host cell damage, which was in stark contrast to equivalent experiments with *S. aureus*, where internalisation was more efficient, but host cell death occurred after just 72 h (Tamilselvam *et al.*, 2006). Microscopy was used to visualise *S. uberis* cells residing within vacuoles in epithelial cells but also surviving freely in the cytoplasm (Matthews *et al.*, 1994a).

Adhesion of ECMP to SUAM corresponded to increased internalisation of *S. uberis* into mammary cells (Almeida *et al.*, 1999b; Almeida *et al.*, 2006). To enter host cells, *S. uberis* appears to exploit host cell protein kinases, phosphorylating these surface proteins to instigate signal transduction pathways which result in microfilament rearrangement of the host actin cytoskeleton (Almeida *et al.*, 2000; Matthews *et al.*, 1994a). Similarly, host cell protein kinases are utilised to mediate invasion of *S. aureus* and *E. coli* into host cells (Agerer *et al.*, 2003; Dopfer *et al.*, 2001). Recent *in vitro* research demonstrated that *S. uberis* also exploits caveolae-mediated endocytosis preferentially to receptor-mediated endocytosis to promote uptake into non-phagocytic cells (Almeida and Oliver, 2006). Moreover, in a strain derived from a chronic, persistent infection, increased internalisation and survival in epithelial cells was demonstrated, this being considerably reduced when caveolae-mediated endocytosis was inhibited (Almeida and Oliver, 2006; Tamilselvam *et al.*, 2006). Observations

regarding adhesion and internalisation have yet to be characterised in multiple strains of *S. uberis* to determine the relative importance and conservation of individual mechanisms; however, the gene encoding SUAM (*sua*), was identified in twelve distinct *S. uberis* strains suggesting it may be important for survival (Luther *et al.*, 2008).

1.4.3 Evading host defences

As with most sites within the mammalian host, the mammary gland is capable of mounting an immunological response against invading pathogens. Consequently, bacteria which invade the mammary gland will be rapidly destroyed by host immune cells (phagocytes) unless they have adapted mechanisms to avoid this process. The role of bacterial exopolysaccharide capsules in resistance to phagocytosis and thus virulence has been demonstrated frequently with streptococci including *S. pyogenes* (Moses *et al.*, 1997; Wessels *et al.*, 1991), *Streptococcus milleri* (Kanamori *et al.*, 2004) and *Streptococcus suis* (Brazeau *et al.*, 1996; Segura *et al.*, 2004). The capsule functions by suppressing phagocytic activity through masking of bacterial antigens, binding and depletion of opsonic factors, providing a physical barrier between the bacteria and phagocyte and inducing electrostatic repulsion. In addition to phagocytic evasion, *S. pyogenes* bacterial capsule has been shown to improve adherence of bacteria to alveolar epithelial cells (Okamoto *et al.*, 2004), and promote adherence to keratinocytes via an M-protein-independent pathway (Schrager *et al.*, 1998). A potential role for capsule in the pathogenesis of *E. coli* has also been shown as encapsulated strains caused more severe mastitis than acapsular strains (Hill, 1981).

Capsule production by strains of *S. uberis* was similarly observed, and the capsule shown to be composed of hyaluronic acid (Almeida and Oliver, 1993b). The capsule of *S. uberis* was shown, *in vitro*, to assist in the evasion of phagocytosis by bovine macrophages with capsule production being stimulated in the presence of milk whey (Almeida and Oliver, 1993a; Almeida and Oliver, 1993b; Almeida and Oliver, 1995). As with *S. pyogenes*, the *S. uberis* capsule has also been suggested to play a role in adherence to ECMP (Almeida *et al.*, 1996). Capsular *S. uberis* strains were shown to be non-antigenic, with cells being visualised in direct contact with macrophage membranes with no signs of membrane activation (Almeida and Oliver, 1993a). It has been estimated that approx. 50 % of *S. uberis* isolates produce hyaluronic acid capsules, but as capsules are not required for proliferation during *in vitro* subculture they are frequently lost, making accurate quantification difficult.

The synthesis of hyaluronic acid capsule by *S. pyogenes* involves hyaluronate synthase, UDP-glucose dehydrogenase and UDP-glucose pyrophosphorylase which were found to be encoded by *hasA, hasB* and *hasC* genes respectively (Crater and van de Rijn, 1995; DeAngelis *et al.*, 1993; Dougherty and van de Rijn, 1993; Dougherty and van de Rijn, 1994). Disruption of *S. pyogenes hasA* correlated with a lack of capsule production, this leading to the discovery that *hasA* was the first gene in an operon which also comprised, in order, *hasB* and *hasC* (Crater and van de Rijn, 1995; Dougherty and van de Rijn, 1994). The *hasABC* operon was found to be controlled by a single transcriptional promoter, lying just upstream of *hasA* (Alberti *et al.*, 1998; Crater and van de Rijn, 1995). Despite the variable ability of strains to produce capsule, the *hasABC* operon was highly conserved amongst even diverse strains of *S. pyogenes* (Wessels *et al.*, 1994). Variability in capsule expression was found to be driven instead by differences in the promoter structure (Alberti *et al.*, 1998). Despite the apparent requirement for all three genes in capsule formation, in *S. pyogenes,* only *hasA* and *hasB* were essential for hyaluronic acid capsule expression, the implication being that an alternative source of UDP-Glucose is available (Ashbaugh *et al.*, 1998).

Capsule formation by *S. uberis* also involves homologues of the *S. pyogenes has* genes, however, the *hasABC* operon structure was not conserved; instead, two discrete loci were identified in strain 0140J, separately harbouring either the *hasAB* or *hasC* genes (Ward *et al.*, 2001). In clinical and environmental isolates, two genotypes were identified, those harbouring the *hasAB* and *hasC* genes and those with just the *hasC* gene; isolates amplifying all three genes were predominant, particularly from clinical isolates (Field *et al.*, 2003). Isolates lacking the *hasA* gene have since been derived more frequently from cases of bovine mastitis, although, there was a trend for isolates from the dominant sequence types (STs) to carry *hasA* over less frequently isolated STs (Coffey *et al.*, 2006; Tomita *et al.*, 2008). Disruption of *hasA* or *hasC* genes produced acapsular mutants with reduced resistance to bovine neutrophil phagocytosis *in vitro*, demonstrating the requirement for both genes during capsule formation (Ward *et al.*, 2001). Despite these *in vitro* observations, in a challenge experiment similar levels of inflammation and equivocal bacterial shedding were caused by both the mutant and the capsular parent strain, implying virulence was not affected by loss of capsule (Field *et al.*, 2003). This was in contrast to similar experiments with *S. pyogenes* where virulence of an acapsular mutant was significantly reduced (Wessels *et al.*, 1991). Furthermore, treatment of capsular strains of *S. uberis* with hyaluronidase did not increase their susceptibility to bovine neutrophils during *in vitro* growth in the presence of casein (Leigh and Field, 1991). Antibodies from immune sera also bound equally to capsular

and non-capsular strains (Leigh and Field, 1994). Taken together, these results imply that the hyaluronic acid capsule alone does not increase resistance of *S. uberis* cells to phagocytosis, and thus production is clearly not essential for the development of mastitis. Additional, unknown, potentially extracellular factors are hypothesised to contribute to resistance of *S. uberis* to phagocytosis (Field *et al.*, 2003).

1.4.4 Nutrient acquisition and dissemination

Bacterial toxins can cause significant damage to host cells. While serving as a means of nutrient acquisition, they can also facilitate the dissemination of bacteria from the primary site of infection, and can thus enhance the severity of infection. Toxins produced by bacteria pathogenic to cattle have been well studied, predominantly because of links to food poisoning in humans, rather than for their contribution to disease progression in the animal host (Fremaux *et al.*, 2006; Niskanen *et al.*, 1978). Isolates of *E. coli* and *S. aureus* from cases of bovine mastitis have frequently been identified as toxin producers (Kenny *et al.*, 1992; Kenny *et al.*, 1993; Srinivasan *et al.*, 2006; Stephan and Kuhn, 1999). Whilst no toxins, *per se*, have been identified with a clear role in the pathogenicity of *S. uberis*, secreted proteins with similar functions for nutrient acquisition and dissemination have been described.

1.4.4.1 Hyaluronidase

Hyaluronidase is an enzyme which cleaves hyaluronic acid, a GAG which is ubiquitous in the mammalian extra cellular matrix (ECM). Hyaluronidase is thus able to digest host connective tissue and facilitate the spread of bacterial infection. Consequently, it is perhaps not surprising, that all Gram-positive bacteria which have been found to secrete hyaluronidase are pathogenic (Girish and Kemparaju, 2007). Extracellular and intracellular, bacteriophage associated hyaluronidase genes have been identified from *S. pyogenes* (Hynes *et al.*, 1995; Hynes *et al.*, 2000). Disruption of the *S. pyogenes* extracellular hyaluronidase-encoding gene (*hylA*) did not, however, affect the spread of the bacteria or the severity of skin lesions in a mouse model, in comparison to the wild-type strain which was capable of digesting hyaluronic acid (Starr and Engleberg, 2006). Conversely, hyaluronidase production by *Streptococcus pneumoniae* was highly associated with disease isolates rather than healthy carriers, and in a mouse infection model facilitated dissemination across the blood/brain barrier (Kostyukova *et al.*, 1995). Similarly, a hyaluronidase deficient mutant of *S. aureus* was less virulent than the wild-type strain (Makris *et al.*, 2004). An entire panel of *S. uberis* isolates were found to produce free hyaluronidase (Schaufuss *et al.*, 1989) but very little research has been conducted in this area since, and hyaluronidase production has not, as yet, been

correlated to dissemination. Hyaluronidase from *S. uberis* was shown however, to inhibit mammary epithelial cell proliferation, although not as efficiently as hyaluronic acid capsule (Matthews *et al.*, 1994b). Recently, it was determined that no homologues of any of the *S. pyogenes* hyaluronidase genes are present in the genome of *S. uberis* 0140J, and thus it was concluded that hyaluronidase carriage amongst streptococci is largely determined by bacteriophage (Ward *et al.*, 2009).

An alternative role for secreted hyaluronidase in nutrient acquisition has also been suggested following observations that growth of hyaluronidase producing *S. pyogenes* isolates was permitted in minimal medium supplemented with hyaluronic acid as the only carbon source (Starr and Engleberg, 2006). As hyaluronidase activity amongst bovine *S. agalactiae* mastitis isolates was significantly higher than amongst human isolates, it can also be implied that hyaluronidase plays an important, but as yet undefined role in mastitis pathogenesis. Further work towards defining the contribution of hyaluronidase to *S. uberis* pathogenesis is thus clearly required.

1.4.4.2 CAMP Factor

The CAMP factor, often alternatively termed co-hemolysin, which was named after its discoverers, Christie, Atkins and Munch-Petersen has also been considered to be a virulence factor produced by *S. uberis*. The CAMP factor is a secreted protein that was originally described in *S. agalactiae*, which, in the presence of *S. aureus* β–toxin, causes the synergistic lysis of sheep erythrocytes through the formation of pores within the membrane (Christie *et al.*, 1944; Lang and Palmer, 2003). A similar protein to CAMP factor, sometimes also called "uberis-factor", has been reported to be excreted by some strains of *S. uberis* (Lammler, 1991; Skalka *et al.*, 1980).

The structural gene for the *S. uberis* CAMP factor has been identified and the translated sequence was found to be homologous to the *S. agalactiae* CAMP protein (Jiang *et al.*, 1996), with antibodies against the *S. uberis* factor also found to cross-react with the *S. agalactiae* factor (Jiang *et al.*, 1996). The toxicity of the *S. uberis* CAMP factor was demonstrated by the intravenous administration of a single dose of the exosubstance (1,500 activity units/kg body weight) to rabbits, which caused rapid death; mice were similarly affected although a dose 45 times greater was required to cause death (Skalka and Smola, 1981). Subsequently, it has been demonstrated, however, that the endogenous *S. agalactiae* CAMP factor is not essential for systemic virulence in a mouse model (Hensler *et al.*, 2008). A role for *S. agalactiae* CAMP factor in resistance to phagocytosis has also been postulated, based upon the ability of the protein to bind in a

non-specific manner to immunoglobulins (Jurgens *et al.*, 1987). An *S. agalactiae* mutant, deficient in the production of CAMP factor was, however, no more susceptible to phagocytosis, *in vitro*, by bovine PMNs, than the isogenic wild-type parent strain (M. Fontaine, unpublished data). The exact contribution of this protein to *S. agalactiae* virulence is thus yet to be confirmed. Similarly, the contribution of the CAMP protein to the pathogenicity *of S. uberis* remains undefined.

Besides the more obvious attributes associated with bacteria that are able to persist within the udder, such as their ability to successfully invade the gland and resist destruction by host immune cells, there is also a more mundane aspect, which affects all organisms, pathogenic or otherwise; the acquisition of nutrients is an absolute requirement to maintain viability and support proliferation. Significantly, it is often the process of nutrient acquisition that contributes to host tissue damage, giving rise to the disease-associated pathology. *S. uberis* is auxotrophic for the amino acids arginine, glutamic acid, histidine, isoleucine, leucine, methionine, tryptophan and valine (Kitt and Leigh, 1997), and therefore must acquire these from the host environment. Other essential nutrients include carbohydrates, vitamins and trace elements such as iron, manganese and zinc which are generally regarded to be necessary for bacterial growth. Consequently, following millennia of co-evolution, the mammalian host has developed mechanisms of limiting bacterial infection through the ability of the innate immune system to limit the availability of utilisable nutrients.

1.4.4.3 Plasminogen activator

Plasminogen, present in blood plasma, is a pro-enzyme precursor of the serine protease plasmin, which degrades blood plasma proteins. Plasmin has also been identified as a principal protease of normal milk, and cleaves milk proteins, especially casein and lactoferrin to their constituent peptides (Andrews, 1983; Aslam and Hurley, 1997). In mastitic milk, plasmin is highly associated with PMNs and it is believed that proteases enhance their phagocytic efficiency (Mehrzad *et al.*, 2005; Reeves *et al.*, 2002). Milk plasmin levels thus increase significantly during episodes of mastitis (Mehrzad *et al.*, 2005), presumably in response to attempts by the host to clear invading pathogens by phagocytosis.

Plasminogen activators control plasmin levels through the conversion of inactive plasminogen into the active protease plasmin. Plasminogen activators in bovine milk are associated with milk somatic cells, and increases in somatic cell numbers corresponded with a large increase in plasminogen activator activity per cell (White *et al.*, 1995;

Zachos *et al.*, 1992). Consequently, plasminogen activator activity is enhanced during inflammation, increasing the levels of plasmin in mastitic milk (Zachos *et al.*, 1992). Despite increased plasmin levels, which would be expected to improve phagocytosis by PMN and thus assist bacterial clearance, some bacteria have been shown to proliferate in milk when plasmin levels are increased (Marshall and Bramley, 1984). Improved growth of the weakly proteolytic-auxotroph *Streptococcus thermophilus* in mastitic milk, was, for example, attributed to plasmin-mediated breakdown of inaccessible milk caseins to accessible amino acids or peptides (Marshall and Bramley, 1984).

Many bacteria have now been identified as producing plasminogen activators to increase plasmin-mediated proteolysis for acquisition of additional nutrients from a limited environment. Plasminogen activators may thus have an important role in virulence. This hypothesis is supported by observations that the streptococcal plasminogen activator, streptokinase, is highly specific for human plasminogen and is conserved among many streptococci pathogenic to humans (Frank *et al.*, 1995; Leigh, 1993). The degradation of ECM molecules and subsequent induction of vascular leakage caused by the production of streptokinase has been shown to contribute to the pathogenicity of *S. pyogenes* and *Streptococcus mutans* by enhancing tissue invasion and thus the severity of infection (Jones and Holt, 2004; Khil *et al.*, 2003; Sun *et al.*, 2004a; Svensson *et al.*, 2002).

In *S. uberis,* the ability to activate plasminogen was first observed in 1993, and this activity was shown to be specific for bovine, ovine and equine plasminogen. The active protein was termed PauA for plasminogen activator uberis A (Leigh, 1993; Rosey *et al.*, 1999), or SUPA for *S. uberis* plasminogen activator (Sazonova *et al.*, 2001). So far, PauA is found exclusively in *S. uberis* and has limited homology to other streptokinases (Frank *et al.*, 1995; Johnsen *et al.*, 1999; Rosey *et al.*, 1999; Ward and Leigh, 2004). The *pauA* gene was identified, and high sequence conservation demonstrated, amongst all *S. uberis* isolates tested, with just one exception; a mastitis-causing strain isolated from a cow in Denmark (Johnsen *et al.*, 1999; Ward and Leigh, 2002; Ward and Leigh, 2004). This Danish isolate was subsequently found to produce a larger, novel plasminogen activator, termed PauB and the *pauB* gene was observed to have directly replaced *pau*A within the chromosome (Ward and Leigh, 2002; Ward and Leigh, 2004). Subsequently, PauB has been shown to bind plasminogen from a considerably broader host spectrum, than PauA, including ovine, bovine, porcine and human (Ward and Leigh, 2002).

The frequency of carriage and high sequence conservation of *pau*A suggests that PauA may have an important role in *S. uberis* virulence, just as streptokinase has been shown

to be important in other streptococcal species. A mutant in which *pau*A was disrupted was still able to survive in milk however, and caused infection at levels similar to the parent strain, suggesting that *pauA* is actually not essential for survival of *S. uberis in vivo* (Ward *et al.*, 2003). Subsequently, a further two *pauA* negative *S. uberis* isolates have been described (Zadoks *et al.*, 2005a). *S. uberis* must therefore have the ability to utilise additional mechanisms for the acquisition of essential nutrients, and the specific role PauA plays in the overall pathogenicity of *S. uberis* still remains unclear.

Bacterial plasminogen activators have also been shown to activate plasmin for the destruction of ECM constituents such as fibronectin (Jones and Holt, 2004). The ECM restricts the movement of bacteria between defined compartments, thus, it is hypothesised that plasmin may enhance the dissemination of bacteria during infection (Klempner *et al.*, 1995). Further studies are required to substantiate this idea and to date there is no evidence supporting this hypothesis in *S. uberis*.

1.4.4.4 Oligopeptide transport system

Free peptides within the extra-cellular environment, liberated by the digestion of host proteins, can only be utilised as a nutrient source if a system exists for their uptake into cells. It has been postulated that *S. uberis* uses an oligopeptide transport system for the acquisition of peptides, similar to that essential to the growth of *Lactococcus lactis* in milk (Smith *et al.*, 2002; Tynkkynen *et al.*, 1993). This system requires the *oppDFBCA* genes which encode ATP-binding, membrane and substrate-binding proteins (Tynkkynen *et al.*, 1993). An *S. uberis* mutant was identified with an alteration in a gene homologous to the *oppF* gene of *S. pyogenes* and this mutant could not acquire amino acids from plasmin-derived casein peptides (Smith *et al.*, 2002). Two genes encoding *oppA*-like binding proteins were also up-regulated during growth of *S. uberis* in milk (Taylor *et al.*, 2003). These results would suggest that some form of oligopeptide transport system is utilised by *S. uberis* as a method for obtaining amino acids. It is important to note however, that both mutants remained viable despite marked reductions in growth (Smith *et al.*, 2002; Taylor *et al.*, 2003), suggesting that once again *S. uberis* does not rely solely on this mechanism for the acquisition of essential nutrients.

1.4.4.5 Aerobic respiration, anaerobic respiration and fermentation

The mechanisms utilised by an organism to generate energy and remove subsequent toxic by-products and waste is dependent upon its repertoire of enzymes and coenzymes as well as the availability of nutrients. Glycolysis, in which glucose is

converted to pyruvate with a net production of 2 molecules of adenosine triphosphate (ATP), is the first stage of both respiration and fermentation. In the presence of oxygen, pyruvate is oxidised to acetyl-coA and fed into the Krebs cycle, generating CO_2 and reduced nicotinamide adenine dinucleotide (NADH). Oxidative phosphorylation of the generated NADH synthesises further ATP by electron transport, in which O_2 is required as the terminal electron acceptor. In the absence of oxygen, pyruvate is converted to waste products, such as lactic acid (fermentation), which are removed from the cell, oxidising the electron carriers and allowing them to be re-used for glycolysis. Alternatively, anaerobic respiration utilises inorganic compounds such as nitrate, iron or sulfate as the final electron acceptor of the electron transport chain.

S. uberis is considered a facultative anaerobe, thus can switch between aerobic and anaerobic metabolism, however, streptococci chiefly produce lactic acid by fermentation of sugars (Mundt, 1982). Despite aerobic respiration having the potential to generate considerably more energy, as long as sugars are readily available for consumption, fermentation may still be carried out in the presence of oxygen. One study demonstrated that *S. uberis* displays a preference for fermentation, consuming less oxygen and converting more glucose to lactic acid than *S. dysgalactiae* and *S. agalactiae* (Mickelson and Brown, 1985). Analysis of the *S. uberis* 0140J genome demonstrated that, like *S. agalactiae*, the respiratory chain is incomplete, lacking in quinone and haem which are components/cofactors of the electron transport chain (Ward *et al.*, 2009). Addition of exogenous haem and quinone to *S. agalactiae* cultures, and the subsequent shift to aerobic respiration, significantly altered the bacterial growth characteristics, with biomass being doubled (Yamamoto *et al.*, 2005). Equally, for *S. uberis* 0140J to undergo aerobic respiration, these elements must be acquired from an alternative source. Considering that the niches inhabited by *S. uberis* are typically also resident to an array of additional organisms, it has been speculated that, acquisition of the haem and quinone products of other bacteria, such as *Lactococcus*, is not unlikely (Ward *et al.*, 2009). Indeed it has been demonstrated that *L. lactis* donates quinones to *S. agalactiae* supporting cooperative behaviour, and, in the presence of exogenous haem, this activates the respiratory chain of *S. agalactiae* stimulating growth (Rezaiki *et al.*, 2008). At this stage, it is not known, however, whether quinone and haem are similarly absent from additional strains of *S. uberis*.

The normal mammary gland is microaerophilic, whilst the inflamed gland is anaerobic with oxygen tension values of 3.11 KPa and 0.17 KPa respectively (Goldberg *et al.*, 1995; Mayer *et al.*, 1988). There was no difference in the growth rates of *E. coli* under

both these conditions (at 39 ^{o}C), replicated in an artificial intramammary environment; although under anaerobic conditions bacteria were not destroyed by bovine neutrophils as they were in the presence of oxygen (Goldberg *et al.*, 1995). Intracellular survival of *S. aureus* in bovine neutrophils was similarly greater under anaerobic over aerobic conditions (Mayer *et al.*, 1988).

The availability (or lack) of oxygen may also influence bacterial virulence. For example, in the presence of oxygen, *S. agalactiae* invaded immortalised human epithelial cells more successfully and was more virulent in a mouse model than when equivalently grown without oxygen (Johri *et al.*, 2003). Capsular polysaccharide (CPS) production by opaque variants of *S. pneumoniae* (which produce more CPS than transparent variants of the same strain and thus are more resistant to phagocytosis), was also influenced by oxygen availability, in this case being significantly reduced as the oxygen concentration was increased, reducing bacterial virulence (Weiser *et al.*, 2001). Further work is required to determine the impact of oxygen upon the virulence of *S. uberis* and any contributing role this may play in mastitis development.

1.4.4.6 Metal transporter uberis A (MtuA)

Metal ions are fundamental to bacterial survival; therefore, bacteria express metal-binding proteins in an attempt to obtain ions which are limited in their environment (He *et al.*, 2006; Khan *et al.*, 2007). A metal binding protein has thus unsurprisingly been identified in *S. uberis* and is referred to as metal transporter uberis A, MtuA (Smith *et al.*, 2003). A mutant in whom the *mtuA* gene was disrupted was unable to grow in bovine milk until manganese was added; this result suggesting that MtuA is specific for binding and transporting manganese (Smith *et al.*, 2003). The importance of manganese acquisition for the growth of *S. uberis* was demonstrated in a challenge model where, unlike the parent strain, the MtuA mutant failed to induce mastitis (Smith *et al.*, 2003). The importance of manganese in intracellular survival has similarly been observed in *Porphyromonas gingivalis* and a role for manganese in protection against oxidative stress was identified (He *et al.*, 2006). These observations imply that MtuA would be a good vaccine candidate, however, the metal binding protein was found to be located within the cell membrane rather than exposed on the cell surface; antibodies against MtuA were thus unable to bind and recognise the bacteria (Jones *et al.*, 2004).

1.5 Mastitis Vaccines

1.5.1 Host defences and immunity in the mammary gland

The development of vaccines against mastitis-causing pathogens is highly complicated due to the nature of the condition itself; this has been the focus of several comprehensive reviews (Anderson, 1978; Denis *et al.*, 2009; Mellenberger, 1977; Yancey Jr., 1993). Mastitis is defined as inflammation of the mammary gland, but inflammation arises following the stimulation of the innate host immune response as it attempts to eliminate the invading pathogen. Further stimulating the host immune response by vaccination to assist in combating future mastitis infections is thus, in itself, likely to create an episode of mastitis, increasing the milk SCC above acceptable levels so that milk has to be discarded at significant financial cost. Thus, unless vaccination offers outstanding subsequent protection, the benefits will not outweigh the costs. The effectiveness of mastitis vaccines is further impeded as a result of the large volumes of milk and vast surface area of the epithelium within the mammary gland, which dilutes immune components. Modification of the host antibody response may improve immunity; however, before serum antibodies can pass into the milk, inflammation is required, thus retarding the ability of antibodies to act in the early stages of infection. Finally, the vast diversity of mastitis pathogens means that a single vaccine is unlikely to combat all mastitis infections, and indeed variation amongst strains of the same species may prevent even a pathogen-specific vaccine being completely effective. This highlights a great need for extensive and detailed characterisation of mastitis isolates.

As reviewed in detail previously (Craven and Williams, 1985), in combination with an understanding of the pathogen, additional knowledge relating to host defence mechanisms is required for effective immunisation. In cattle, innate host defences include both anatomical barriers intended to discourage entry of bacteria into the gland cistern, and cellular defences which are stimulated by the presence of bacteria in the gland. Upon infection, macrophages, resident in the normal mammary gland, release inflammatory mediators, such as cytokines, which in combination with direct stimuli from pathogens, recruit leukocytes (predominantly PMNs) into the mammary gland from the blood (Kaneko *et al.*, 1964; Paape *et al.*, 2000). This vast, non-specific inflammatory cell response can comprise up to 50 million cells per millilitre of milk (Paape *et al.*, 1979). The speed and efficiency of the leukocyte response, and subsequent bacterial phagocytosis has been directly linked to the severity of mastitis episodes induced by *E. coli* (Hill, 1981; Jain *et al.*, 1971; Kremer *et al.*, 1993).

Despite observations that the phagocytic efficiency of milk neutrophils and macrophages is poor due to the indiscriminate ingestion of casein and milk fat globules (Paape and Wergin, 1977; Paape *et al.*, 1979), it is generally accepted that neutrophil chemotaxis and phagocytosis contributes to the control of *E. coli* and *S. aureus* mastitis infections (Grommers *et al.*, 1989; Jain *et al.*, 1971). In an experimental infection, after a lag phase of 48 h, growth of *S. uberis* in milk increased rapidly whilst the SCC peaked before this time point, suggesting that despite host recognition, the resulting leukocyte response was insufficient to prevent growth (Rambeaud *et al.*, 2003). It has further been demonstrated *in vitro* that under specific conditions, particularly in the presence of hydrolysed casein, *S. uberis* can resist the action of bovine neutrophils (Leigh and Field, 1994). Despite the substantial inflammatory response elicited following infection with *S. uberis* (Finch *et al.*, 1997; Pedersen *et al.*, 2003), the importance of neutrophils in controlling infections is thus clearly debateable. This was further implied following pathological observations from glands infected with *S. uberis* which showed bacterial cells were phagocytosed by macrophages but not neutrophils (Thomas *et al.*, 1994); suggesting that macrophages may have a more significant role during infection. It was later demonstrated however that *S. uberis* cells were found within neutrophils of infected mammary glands (Pedersen *et al.*, 2003) and milk macrophages from lactating cows were similarly inefficient at killing *S. uberis* which were able to multiply within these cells (Denis *et al.*, 2006). The importance of cytokines and complement for the recruitment of leukocytes and mediation of phagocytosis in response to *S. uberis* also appears to be limited (Grant and Finch, 1997; Rambeaud *et al.*, 2003). The acute phase protein, serum amyloid A, was evident in infected milk by 10 h post inoculation with *S. uberis* (Pedersen *et al.*, 2003) so it could be speculated to play a role in recruitment of immune cells.

Taking the above into account, it is questionable whether vaccination-induced modification of the inflammatory neutrophil response within the udder is sufficient to offer protection against *S. uberis* mastitis; furthermore, it is possible that such modification may indeed contribute to the condition. As stated, the negative impact of any mastitis vaccine, in terms of increased SCC contributing to rejection of milk, must be weighed against the impact of repeated infection by a given pathogen.

1.5.2 *S. uberis* vaccine research

Commercial mastitis vaccines are available, although these vary in their efficacy, and none targeting *S. uberis* currently exist. The need for novel preventative measures against this pathogen, which is particularly common in the UK and New Zealand (Bradley

et al., 2007; McDougall *et al.*, 2004), are clear, especially considering that good hygiene measures and antibiotic therapy have been shown to be ineffective at controlling mastitis resulting from infection by this pathogen (Milne *et al.*, 2005; Watts, 1988). *S. uberis* has been the target of numerous experimental vaccines over the years, and although results have been variable, none of these vaccines has yet found its place on the market.

In one study, clinical mastitis in challenged quarters was reduced from 87 % following initial inoculation, to 32 % after secondary challenge with *S. uberis*, demonstrating that vaccination against mastitis was possible (Hill, 1988b). Subsequently, both live and killed whole-cell *S. uberis* vaccines have been shown to significantly reduce bacterial numbers, SCCs and clinical signs of mastitis during subsequent challenge (Finch *et al.*, 1994; Hill *et al.*, 1994). Importantly, protection following vaccination was achieved in the absence of a notable neutrophil response (Finch *et al.*, 1994; Hill *et al.*, 1994; Leigh and Field, 1994). Titres of *S. uberis*-specific antibodies (IgG1, IgG2 and IgM) in bovine serum and milk also increased following vaccination with killed bacterial cells (Finch *et al.*, 1994; Leigh and Field, 1994), and milk from challenged mammary glands inhibited growth of the homologous strain compared to unchallenged milk (Fang *et al.*, 1998). Inclusion of a cell extract booster during the vaccination regime only marginally increased the specific antibodies produced but did reduce SCC and bacterial numbers (Finch *et al.*, 1994; Finch *et al.*, 1997).

Protection in vaccinated quarters against homologous challenge with *S. uberis* appeared, however, to be unrelated to mediation of increased phagocytosis by opsonic antibodies or complement (Finch *et al.*, 1994; Hill *et al.*, 1994; Leigh and Field, 1994). Bovine neutrophils were able to destroy *S. uberis*, *in vitro*, in the presence of bovine serum, but this ability was not improved when hyper-immune serum was used over pre-vaccination serum, despite specific antibodies being shown to bind to the bacteria (Leigh and Field, 1994). This suggests that *S. uberis* specific antibodies do not contribute to opsonisation and phagocytosis by bovine neutrophils. The method of homologous protection was thus speculated to involve the reduction of *S. uberis* growth rate in challenged quarters (Finch *et al.*, 1994; Finch *et al.*, 1997). Disappointingly, despite whole-cell *S. uberis* vaccination offering high levels of protection upon repeat infection with the homologous strain (through an undefined mechanism), limited protection was imparted upon glands inoculated with a heterologous strain (Finch *et al.*, 1997). As high genetic diversity has been identified among *S. uberis* isolates (Phuektes *et al.*, 2001), a vaccine which cannot provide heterologous protection has severely

limited value. Contrastingly, echoing findings with *S. aureus*, one study found that serum from vaccinated cows not only permitted opsonisation of capsular strains, but that phagocytosis of heterologous capsular strains was also mediated (Almeida and Oliver, 1995; Guidry *et al.*, 1991). No subsequent corroboration of these observations could, however, be found.

Vaccination against *S. uberis* mastitis has been achieved without stimulating an exuberant inflammatory response, a decrease in milk yield and without causing symptoms of clinical mastitis to develop. Protection was, however, only offered against subsequent infection with the same strain. The identification of specific *S. uberis* antigens which could be used to develop a sub-unit vaccine has thus become the main research focus, as such a vaccine could provide cross protection amongst diverse strains. Virulence factors are often considered to be suitable vaccine candidates because they facilitate bacterial survival and thus are likely to be conserved among pathogenic strains.

The Plasminogen activator (PauA), as discussed earlier, has been described as an important *S. uberis* virulence factor which is believed to function through the acquisition of nutrients and enhancement of dissemination (Jones and Holt, 2004; Marshall and Bramley, 1984). The high conservation of *pauA* among *S. uberis* mastitis isolates (Ward and Leigh, 2004) prompted its evaluation as a vaccine candidate (Leigh *et al.*, 1999). Prior to heterologous challenge, vaccination of cattle with PauA-containing supernatant from cultured *S. uberis*, prevented clinical mastitis in half of the challenged quarters, whilst in contrast, no quarters vaccinated with PauA-depleted supernatant were protected from mastitis (Leigh *et al.*, 1999). Serum IgG from PauA-vaccinated animals showed increased ability to inhibit plasminogen activation by PauA (Leigh *et al.*, 1999). Vaccination reduced but did not prevent bacterial growth, similar to results following whole cell vaccination, thus it was again assumed that protection was attributable to a reduction in the rate of gland colonisation and was not related to neutrophil phagocytosis (Finch *et al.*, 1994; Finch *et al.*, 1997; Leigh *et al.*, 1999). This result was promising, but for protection to be specifically attributed to PauA, purified protein alone, not supernatant, must be used for vaccination, as additional factors may be responsible for the protection seen. Indeed, in a subsequent challenge model, a *pauA* mutant incapable of activating bovine plasmin was no less virulent and induced equivalent clinical mastitis in comparison with the wild-type parent strain (Ward *et al.*, 2003). As PauA is thus clearly not essential for the pathogenesis of *S. uberis*, a vaccine preparation based upon this antigen alone, would likely not be wholly effective.

In additional attempts to develop *S. uberis* mastitis vaccines, the antigens of interest have been; a recombinant *S. uberis* GapC protein (a cell surface associated plasmin receptor homologue) and an engineered CAMP fusion protein (a secreted co-hemolysin) which was intended to offer cross-species protection (Fontaine *et al.*, 2002). Vaccination once again increased specific IgG antibody titres against GapC and CAMP antigens whilst upon subsequent heterologous challenge, decreased SCCs and accelerated recovery of milk quality in vaccinated groups were observed (Fontaine *et al.*, 2002). A reduction in quarters bacteriologically positive for *S. dysgalactiae* was similarly achieved following vaccination with a recombinant GapC protein derived from the same strain (Bolton *et al.*, 2004). Homology between the GapC products of *S. uberis*, *S. agalactiae* and *S. dysgalactiae* prompted the development of a single chimeric protein comprising regions from the proteins of each species, with a view to creating a single vaccine effective against all three mastitis pathogens (Perez-Casal *et al.*, 2004). This antigen has yet to be trialled in a challenge model, but emphasises the potential applications of sub-unit vaccination for offering protection against not only all strains of the same species but also different pathogenic species.

Antibodies against the *S. uberis* adhesion molecule, SUAM, have been shown to inhibit adhesion and internalisation of *S. uberis in vitro* (Almeida *et al.*, 2006). Furthermore, the gene encoding this adhesin is conserved amongst diverse strains (Luther *et al.*, 2008) and thus SUAM may well also prove to be a suitable candidate for sub-unit vaccination. Further research is clearly required for the development of a commercial *S. uberis* mastitis vaccine, but the potential of sub-unit vaccines for heterologous protection has clearly been highlighted. As a reduction in *S. uberis* mastitis would have a significant impact on overall mastitis levels, as well as improving animal health, the impact of a successful *S. uberis* vaccine would be vast.

1.6 Project objectives and aims

The emergence of *S. uberis* as a leading cause of bovine mastitis requires that research be undertaken to support the development of an effective vaccine. In order to develop a successful vaccine, a range of pre-requisites must first be met. A better understanding of the relationships between *S. uberis* strains, and the mechanisms by which they colonise and persist *in vivo* is needed. Based upon typing studies conducted to date (which will be discussed in detail later in this thesis), there is apparently significant heterogeneity between strains of *S. uberis* isolated from cases of mastitis. Combined with the observation that there does not (yet) appear to be any single "magic-bullet"

virulence factor against which an effective sub-unit vaccine can be delivered, the challenges faced in terms of *S. uberis* vaccine development are significant. Consequently, the main objective of the work presented in this thesis was to conduct further, in depth characterisation of *S. uberis* mastitis isolates. The emphasis of this work was not merely to gather new information regarding the relationships between strains, but also to identify novel phenotypes that might be relevant to the ability of the organism to successfully exploit its chosen niche.

Chapter 2: Materials and Methods

2.1 Source of general Reagents

All chemicals used in this study were purchased from Sigma-Aldrich (Poole, Dorset, UK) unless otherwise stated and were of analytical grade. Restriction endonucleases, 25 mM $MgCl_2$, modifying enzymes, molecular weight ladders and 6× loading dye for DNA gels were purchased from Promega UK Ltd (Hampshire, UK). Kits for extraction of DNA or RNA were obtained from Qiagen (West Sussex, UK) unless otherwise stated.

2.2 Sterilisation

Stock solutions of media, media supplements and antibiotics less than 100 ml in volume were sterilised through 0.2 μm or 0.45 μm syringe filters (Sartorius, Epsom, UK). Larger volumes of media, buffers and other heat-tolerant solutions were sterilised by autoclaving at 121 °C for 15 minutes at a pressure of 15 psi. All media, apart from Brain Heart Infusion (BHI) broth were sterilised by autoclaving; BHI broth was sterilised using 0.22 μm filtration units (Corning, Massachusetts, USA).

2.3 Bacteriological techniques

2.3.1 Media preparation and maintenance of bacterial cultures

Media used for cultivating bacteria were prepared in-house from powder (Oxoid, Hampshire, UK) according to the manufacturers' guidelines. Blood agar (BA) plates were prepared by cooling autoclaved blood agar base to approx. 50 °C and supplementing with 5 % (v/v) warmed sheep blood prior to pouring.

S. uberis was routinely cultured and sub-cultured in BHI broth and on BHI or BA plates, which were inoculated using glycerol stocks (**Section 2.3.4**). *E. coli* strains were routinely cultured on Luria Bertani (LB) agar plates or in LB broth medium. Bacteria were cultivated for 24 h at 37 °C, either static (for *S. uberis*) or at 200 rpm (for *E. coli*). Upon receipt, mastitis isolates were resuscitated in 10 ml broth then streaked for single colonies onto solid medium. Where mixed cultures were obtained, a single putative *S. uberis* colony was re-streaked onto a fresh agar plate and a single colony from this plate was used to inoculate an overnight culture. Species identification was confirmed using API tests (**Section 2.3.2**). Anaerobic growth conditions were generated by placing inoculated plates or cultures into a 2.5 l AnaeroJar™ with an AnaeroGen™ sachet

(Oxoid), immediately sealing the jar and then incubating samples as normal. Anaerobic conditions were confirmed using an anaerobic indicator strip (Oxoid) which turns from pink to white when anaerobic conditions are achieved. Strains of *E. coli* and *S. uberis* isolates utilised in this study are listed, with a brief description of their origin, in **Tables 2.1 and 2.2** (with the exception of isolates originating from Italy which are listed in **Chapter 3, Table 3.6**).

S. uberis chemically-defined media (CDM) were prepared either using a complex published recipe (Leigh and Field, 1991), or a novel recipe optimised during this study (the development of which is discussed further in **Chapter 5**). Commercial Roswell Park Memorial Institute (RPMI) medium (Sigma R8755) was utilised as the basal component of the new CDM (**Table 2.3**), which was prepared with or without metal ions, and with lactose or glucose depending upon the requirement of the individual experiment. Into a plastic beaker containing 900 ml of dH_20, 10.39 g RPMI, 44 mM glucose or 22 mM lactose and 1.5 mM L-glutamic acid were added and mixed thoroughly. At this point, if required, 36 μM $FeS0_4.7H_20$, 45 μM $MnS0_4.4H_20$ and 609 μM $MgS0_4.7H_20$ (final concentration) were also added. The pH of the medium was adjusted to 4.0 using 1 M HCl to ensure all reagents were completely dissolved. After pH adjustment, $NaHCO_3$ was added to the medium to a final concentration of 24 mM, and after stirring at room temperature (RT) for approx. 30 m the pH was checked and adjusted to 7.1 with 1 M HCl or NaOH as required. Medium was made up to 1 l with dH_20, sterilised through a 0.2 μm Clyde In-line filter (Whatman, Kent, UK) and stored at 2-8 °C for no more than two months prior to use. Media preparation was completed using plastic-ware only in an attempt to avoid leaching of metal ions from glassware.

To the defined medium, casein (BDH, Dorset, UK) or hydrolysed casein were added as required to a final concentration of 0.5 % (w/v). To more efficiently dissolve casein, medium containing casein was incubated at 37 °C for *ca.* 2 h prior to 0.45 μm syringe filtration. Hydrolysed casein (ch) was easily dissolved in medium at RT and 0.2 μm filter sterilised. Amino acids were also added aseptically to the medium as required, to a final concentration of 1× (v/v), using a commercial 10× amino acid solution. Additional media supplements such as $FeS0_4$, $MnS0_4$ and $MgS0_4$ were prepared as 100 mM stock solutions, whilst the chelating agents, ethylenediamine-N,N'-diacetic acid (EDDA), 2, 2'-dipyridyl and nitrilotriacetic acid (NTA) were prepared as 5 or 10 mM stock solutions. All media

Table 2.1: Bacterial strains and isolates.

ID	Origin	Additional Information
S. uberis		
0140J	Prof. J. Leigh, University of Nottingham, UK	Bovine, ATCC BAA-854. Genome sequence at: http://www.sanger.ac.uk/Projects/S_uberis/ AM946015
20569	DSMZ	Bovine, Type strain Serological group E. ATCC 9436
1.1	US	No additional information
1.3	US	No additional information
1.4	US	No additional information
1.93	US	No additional information
2.97	US	No additional information
2.98	US	No additional information
2.99	US	No additional information
2.100	US	No additional information
E. coli		
TG1-Dev	Prof. M. Kehoe, University of Newcastle-upon-Tyne, UK [A]	Derivative of the common lab strain TG1, carrying the wild-type lactococcal *repA* gene allowing replication of temperature-sensitive plasmids at 37 ºC
BL21/DE3	Commercial – Invitrogen (Renfrew, UK)	One shot chemically competent TOPO

[A] Originally a kind gift of Emmanuelle Maguin, L'Institut National de la Recherche Agronomique (INRA), Jouy-En-Josas, France.

Table 2.2: *S. uberis* isolates from UK cases of clinical bovine mastitis during 2000.

Isolate ID [A]	Farm	Animal ID	Quarter [B]	Sample [C]
T1-3	Baker New Ford	187	FL	O
T1-4	Baker New Ford	187	FL	R
T1-5	Baker New Ford	289	U	S
T1-6	Baker Newton	203	BR	S
T1-9	Barton Brook	122	BR	S
T1-10	Bray	5	BL	S
T1-11	Britton Sella	140	BL	S
T1-13	Bucknell Holways	1847	FL	S
T1-18	Coohe Aller	260	BR	S
T1-20	Crossman Plushayes	60	BL	S
T1-21	Crossman Plushayes	75	BR	O
T1-22	Crossman Plushayes	75	BR	R
T1-23	Crossman Plushayes	75	BR	R
T1-24	Crossman Plushayes	108	BR	O
T1-25	Crossman Plushayes	108	BR	R
T1-26	Daymond V. Dairy	Whiskey	BL	S
T1-27	Derham Channons	160	FL	S
T1-28	Dibble	284	BR	S
T1-29	Dibble	284	FR	S
T1-30	Elston Hotherland	198	FR	S
T1-31	Emmerford	99	FR	S
T1-32	Frank Pitt Rix	755	FR	O
T1-33	Frank Pitt Rix	755	BR	O
T1-34	Frank Pitt Rix	755	BR	R
T1-35	Frank Pitt Rix	755	FR	R
T1-36	Gale Chevithorne	56	BR	O
T1-37	Gale Chevithorne	56	BR	R
T1-38	Gale Chevithorne	56	BR	R
T1-39	Gale Chevithorne	56	BR	R
T1-40	Gale Chevithorne	56	U	U
T1-41	Gale Chevithorne	56	BR	R
T1-42	Gale Chevithorne	56	BR	R
T1-43	Gale Chevithorne	82	FL	S
T1-44	Gilbbins H. Hill	473	FL	S
T1-45	Gilbbins H. Hill	586	BL	S
T1-46	Gilbbins H. Hill	704	FR	R
T1-47	Gilbbins H. Hill	704	FR	R
T1-48	Gilbbins H. Hill	704	FR	R
T1-49	Gilbbins H. Hill	704	FR	O
T1-50	Hann	19	FR	R
T1-51	Hann	19	FR	O
T1-52	Hann GT. Bradley	2	BL	O
T1-53	Hann GT. Bradley	2	BL	R
T1-54	Hann GT. Bradley	13	FR	O
T1-55	Hann GT. Bradley	13	FR	R
T1-56	Harris Southwood	54	FR	S
T1-57	Hayball	Olive	BR	S
T1-58	Headon Huntlands	34	BL	S
T1-59	Hellier	Camex	FL	S
T1-60	Hellier Whit	Carmel	BL	S
T1-61	Hellier	Jackie	BL	S
T1-62	Henson	341	FR	S
T1-63	Hill Fulford	2	BR	S

T1-64	Hill Fulford	42	BR	S
T1-65	Hill Fulford	99	FL	S
T1-66	Hill Fulford	125	BR	S
T1-67	Hill	68	U	S
T1-68	Horrell Bridge	240	BL	S
T1-69	Horrell Bridge	617	U	S
T1-70	Hughes - Clares Barn	1	FR	O
T1-71	Hughes - Clares Barn	1	FR	R
T1-72	Hughes - Clares Barn	31	FL	O
T1-73	Hughes - Clares Barn	31	FL	R
T1-74	Hughes - Clares Barn	42	BR	S
T1-75	Hughes - Clares Barn	71	BL	S
T1-76	Hughes - Clares Barn	75	BL	O
T1-77	Hughes - Clares Barn	75	BL	R
T1-78	Hughes - Clares Barn	75	BL	R
T1-79	Hughes - Clares Barn	127	BL	S
T1-80	Crossman Plushayes	108	BR	R
T1-81	Crossman Plushayes	108	BR	O
T2-1	Hughes - Clares Barn	157	FR	S
T2-2	Hughes - Clares Barn	182	FR	O
T2-3	Hughes - Clares Barn	182	FR	R
T2-4	Hughes - Clares Barn	182	BR	S
T2-5	Hughes - Clares Barn	195	BL	O
T2-6	Hughes - Clares Barn	195	BL	R
T2-7	Hughes - Clares Barn	195	BL	R
T2-9	Hughes - Clares Barn	201	FR	S
T2-10	Hughes - Clares Barn	266	FL	O
T2-11	Hughes - Clares Barn	266	FL	R
T2-12	Hughes - Clares Barn	266	FL	R
T2-13	Hughes - Clares Barn	266	FL	R
T2-14	Hughes - Clares Barn	266	FL	R
T2-15	Hughes - Clares Barn	279	BR	S
T2-16	Hughes - Clares Barn	293	FL	O
T2-17	Hughes - Clares Barn	293	FL	R
T2-18	Hughes - Clares Barn	293	FL	R
T2-19	Hughes - Clares Barn	293	FL	R
T2-20	Hughes - Clares Barn	294	BL	S
T2-22	Hughes - Clares Barn	317	FL	S
T2-23	Hughes - Clares Barn	326	FR	S
T2-24	Hughes - Clares Barn	341	BL	S
T2-25	Hughes - Clares Barn	351	BL	S
T2-26	Hughes - Clares Barn	363	FL	S
T2-27	Hughes - Clares Barn	378	BR	S
T2-28	Hughes - Clares Barn	378	FR	S
T2-29	Hughes - Clares Barn	400	BL	O
T2-30	Hughes - Clares Barn	400	BL	R
T2-31	Hughes - Clares Barn	403	FR	O
T2-32	Hughes - Clares Barn	403	FR	R
T2-33	Hughes - Clares Barn	408	FR	O
T2-34	Hughes - Clares Barn	408	FR	R
T2-35	Hughes - Clares Barn	445	BR	S
T2-36	Hughes - Clares Barn	516	FR	S
T2-37	Hughes - Clares Barn	584	FL	S
T2-38	Hughes - Clares Barn	681	BL	S
T2-39	Hughes - Clares Barn	683	FL	S

T2-40	Hughes - Clares Barn	728	BL	S
T2-41	Hughes - Clares Barn	728	BR	R
T2-42	Hughes - Clares Barn	728	FL	S
T2-43	Hughes - Clares Barn	728	BR	O
T2-44	Hughes - Clares Barn	735	BL	O
T2-45	Hughes - Clares Barn	735	BL	R
T2-46	Hughes - Clares Barn	750	BR	S
T2-47	Hughes - Clares Barn	753	FL	O
T2-48	Hughes - Clares Barn	753	BR	S
T2-49	Hughes - Clares Barn	753	FL	R
T2-50	Hughes - Clares Barn	783	BL	O
T2-51	Hughes - Clares Barn	783	BL	R
T2-52	Hughes - Clares Barn	783	BL	R
T2-53	Hughes - Clares Barn	784	BL	O
T2-54	Hughes - Clares Barn	784	BL	R
T2-55	Hughes - Clares Barn	784	BL	R
T2-56	Hughes - Clares Barn	821	BL	S
T2-57	Hughes - Clares Barn	821	FL	S
T2-58	Harris	92	BR	S
T2-59	Kingdom GT. Hayne	1386	FR	O
T2-60	Kingdom GT. Hayne	1386	FR	R
T2-61	Lee Dalwood	1091	FL	S
T2-62	Lee Dalwood	99	BR	S
T2-63	Lewis Smithincott	160	BR	S
T2-66	Olive LR Coliprest	2	FL	S
T2-67	Paine Easteridge	198	BL	O
T2-68	Paine Easteridge	198	BL	R
T2-69	Paine Easteridge	198	BL	R
T2-70	Paine Easteridge	198	BL	R
T2-72	Paine Easteridge	915	FR	O
T2-73	Paine Easteridge	915	FR	R
T2-74	Persey	349	U	S
T2-75	Persey Fordmore	86	FL	S
T2-76	Persey Fordmore	154	BL	S
T2-78	Persey Fordmore	327	BL	S
T2-79	Persey Langlands	5	FR	S
T2-80	Persey Langlands	402	BR	S
T2-81	Persey Langlands	513	FL	S
T3-1	Persey Park	D72	BL	S
T3-2	Persey Park	L945	FR	S
T3-3	Persey Park	L949	BL	S
T3-4	Persey Park	R181	FL	S
T3-5	Pyle Northill	U	FL	S
T3-6	Pyle Treasebeard	63	BL	S
T3-7	Reed Dungeons	39	FL	S
T3-8	Reed Dungeons	146	BR	S
T3-9	Reed Dungeons	234	BR	S
T3-12	Squire Henland	Dilys	BL	S
T3-14	Stacey Jurishayes	71	BL	S
T3-15	Stacey Jurishayes	136	FL	S
T3-16	Summers Wessington	799	BR	S
T3-17	Summers Wessington	799	BR	S
T3-18	Thomas Ewings	82	BL	S
T3-21	Thomas Ewings	454	FL	S
T3-23	Thomas HR Brithayes	72	BL	S

T3-24	Vallis Highdown	250	FR	S
T3-25	Vallis Highdown	289	FL	S
T3-31	Webber Cranklands	459	FR	S
T3-32	Whitnage	Carne	FL	S

[A] Isolates received from M. Milne, Glasgow, UK (Milne *et al.*, 2002).

[B] Udder quarter designations; back left (BL), back right (BR), front left (FL) and front right (FR) or quarter unknown (U).

[C] Animals with clinical mastitis were treated with veterinary antibiotics and infection either persisted, thus original (O) and replicate (R) samples were obtained from the same animal over time, or the infection was resolved and as such only a single sample (S) was obtained. Where animal quarter information was not know it was not possible to determine if the sample was a replicate isolate from the same animal, or a single isolate from a distinct quarter, so these were marked as unknown (U).

Table 2.3: Composition of RPMI-1640 medium (Sigma, R8755)

Components	g/L
L-Arginine (Free Base)	0.2
L-Asparagine (Anhydrous)	0.05
L-Aspartic Acid	0.02
L-Cystine.2HCl	0.0652
L-Glutamic Acid	0.02
L-Glutamine	0.3
Glycine	0.01
L-Histidine (Free Base)	0.015
Hydroxy-L-Proline	0.02
L-Isoleucine	0.05
L-Leucine	0.05
L-Lysine.HCl	0.04
L-Methionine	0.015
L-Phenylalanine	0.015
L-Proline	0.02
L-Serine	0.03
L-Threonine	0.02
L-Tryptophan	0.005
L-Tyrosine.2Na.2H_2O	0.02883
L-Valine	0.02
Biotin	0.0002
Choline Chloride	0.003
Folic Acid	0.001
myo-Inositol	0.035
Niacinamide	0.001
D-Pantothenic Acid Hemicalcium	0.00025
PABA	0.001
Pyridoxine.HCl	0.001
Riboflavin	0.0002
Thiamine.HCl	0.001
Vitamin B_{12}	0.000005
Calcium Nitrate.4H_2O	0.1
Magnesium Sulfate (Anhydrous)	0.04884
Potassium Chloride	0.4
Sodium Chloride	6.0
Sodium Phosphate Dibasic (Anhydrous)	0.8
D-Glucose	2.0
Glutathione, Reduced	0.001

supplements were prepared in dH_20, sterilised and stored at 2-8 ^oC prior to aseptic addition into medium as required.

Chelex-100® (Chelex) was used to completely remove metal ions from defined medium containing ch. Chelex was added to prepared medium to a final concentration of 2.5 or 5.0 % (w/v). Medium was incubated, with stirring, at RT for up to 20 hours. To remove Chelex matrix and all bound metal ions, the solution was passed through a 1.0 μm pre-filter. The medium was also passed through a 0.2 μm filter for sterilisation. Treated medium was stored in the same manner as untreated medium.

2.3.2 Species identification using API test

The API 20 Strep test kit (bioMérieux, Marcy l'Etoile, France) was used for species confirmation of received *S. uberis* isolates. API tests were completed according to the manufacturer's protocol. Each test isolate was streaked for single colonies onto BA and after incubation at 37 ^oC for approx. 24 h, observations of haemolysis were noted. A single colony was re-suspended in 300 μl dH_20 and after thorough mixing was used to flood a BHI agar plate which was incubated, as above. To prepare the incubation box for the test, 5 ml water was distributed into the bottom of the tray and a test strip was placed into the labelled tray. An ampule of API suspension medium was opened and the entire culture from the flooded plate was collected using a sterile swab and re-suspended in the medium. A turbidity value of greater than 4 McFarland units was required and this was determined roughly by visual comparison to a standard. Aliquots of the suspension were distributed into the wells marked VP to ADH and the remaining suspension was mixed into an opened ampule of API GP medium. The new suspension was distributed into the remaining wells of the strip. Marked tests were covered with a mineral oil layer and after replacing the lid on the tray, test strips were incubated at 37 ^oC for approx. 4 h. The appropriate reagents were added; VP 1 and VP 2 to VP test, NIN to HIP test and ZYM A and ZYM B to remaining wells lacking mineral oil. After 10 m, results were compared to the reference table provided and marked on a results sheet. Interpretation of API results was completed using the bioMérieux programme APILAB v.3.3.3. Identification scores of greater than 90 % were considered acceptable, and scores of 99.9 % were routinely achieved. Further observations were made after 24 h where directed.

2.3.3 Antibiotics

Antibiotics were added to growth media from concentrated stock solutions of 10 or 100 mg/ml when required. Erythromycin stock solutions were prepared by dissolving powder in 100 % ethanol, whilst kanamycin and ampicillin stocks were prepared by dissolving powder in distilled water. Aliquots of 1 ml were prepared and stored at -20 ^{o}C until required.

2.3.4 Bacterial storage

Bacterial strains/isolates were stored as 20 % (v/v) glycerol stocks. Stocks were prepared by aliquoting 1.125 ml of overnight cultures into cryovials containing 375 μl of 80 % (v/v) glycerol. After mixing, glycerol stocks were stored at -70 ^{o}C until required. Glycerol stocks were generally utilised for inoculation of starter cultures for experimental work.

2.3.5 Bacterial growth analysis

The bioscreen C apparatus (Growth curves Ltd.) was used to simultaneously analyse bacterial growth characteristics using 100 well honeycomb plates (Thermo Fisher Scientific, Waltham, Massachusetts) from which readings of optical density (OD) at 600 nm were taken by a spectrophotometer at defined intervals (in this study every 15 m) using a vertical light path. The apparatus was attached to a computer allowing absorbance readings taken at each time point to be recorded. Each medium inoculated was analysed in triplicate and compared to triplicate un-inoculated medium control samples for normalization of results from each media. Stationary, or log phase bacteria (prepared in BHI broth or CDM from glycerol stocks) were used to prepare 100-fold bacterial dilutions into specific medium of interest for analysis. Prior to each reading, plates were shaken for 15 s using the high intensity setting.

The growth data obtained was transferred into a Microsoft Excel spreadsheet. Average absorbance values were calculated for both the inoculated and un-inoculated medium samples at each time point. To normalize results, the average inoculated absorbance value was subtracted from the average un-inoculated absorbance to give a corrected absorbance value. Corrected absorbance values were obtained for each time point allowing construction of a bacterial growth curve. Growth curves obtained in different medium could then be directly compared.

2.4 Plasmid vectors

Plasmids utilised in this study were as follows: Firstly, the Gram-positive shuttle plasmid, pG$^+$host 9 (Maguin *et al.*, 1996), a temperature-sensitive vector encoding an erythromycin resistance marker, was used for targeted allele replacement mutagenesis of *S. uberis*. Similarly, the plasmid pGh9:IS*S1*, a derivative of pG$^+$host 9 containing the IS*S1* insertion sequence (Maguin *et al.*, 1996) was used to create an *S. uberis* random-insertion mutant library. Finally, the commercially available pCR®-Blunt II-TOPO® plasmid was used along with the TOPO kit (Invitrogen) for cloning of blunt end PCR products prior to sub-cloning into pG$^+$host 9.

2.5 Extraction of genomic DNA from *S. uberis*

The NucleoSpin® Tissue Kit and NucleoSpin® 96 Tissue Kit (Macherey-Nagel, Düren, Germany) were used respectively for small and large scale extraction of genomic DNA (gDNA) from *S. uberis* isolates. DNA extraction was conducted using either a vacuum manifold (for 96-well plates) or a bench top micro-centrifuge for individual spin columns. The manufacturer's guidelines for the isolation of DNA from bacteria were followed with some amendments.

To obtain stationary phase cultures, glycerol stocks of *S. uberis* isolates were used to inoculate 2 ml of BHI broth and were incubated for 20 to 24 h. Overnight cultures were centrifuged for 15 m at 2,000 × *g* and cell pellets re-suspended in 300 µl Gram-positive lysis buffer (20 mM Tris-HCl, pH 8.0; 2 mM EDTA; 1 % (v/v) Triton x-100; 2 mg/ml lysozyme). Re-suspended cells were transferred to 1.5 ml micro-centrifuge tubes or 96-well round-bottomed plates and incubated at 37 °C for 30 m. Following incubation, 100 µl of lysate was removed from each well and discarded to avoid overloading the NucleoSpin binding plates or columns. To each tube/well, 2.5 mg/ml Proteinase K was added and samples incubated for 3 h at 56 °C. Samples were vortexed at approx. 15 m intervals during incubation to ensure adequate mixing. For 96-well blocks, after incubation, 200 µl buffer BQ1 and 200 µl 100 % ethanol were added to each well and vortexed vigorously for 15 s. A vacuum manifold was prepared and samples were transferred into the corresponding wells of a binding plate. Vacuum was applied for 10 m after which all lysates had passed through the columns. To individual tubes, 200 µl buffer B3 was added and samples incubated at 70 °C for 10 m before the addition of 200 µl 100 % ethanol. Samples were mixed, transferred to NucleoSpin columns and centrifuged at 14,000 × *g* for 1 m. In both cases column flow-through was discarded and

columns were washed once with 600 μl buffer BW and then twice with either 600 (spin columns) or 900 μl (plates) buffer B5. Spin columns were centrifuged once more to remove all buffer traces; alternatively, the binding plate was removed from the manifold and tapped onto clean paper towels, then returned to the manifold and vacuum applied for a further 10 m to dry the membranes. In both cases, gDNA was eluted with 60 μl of buffer BE, pre-warmed to 70 °C, containing 20 μg/ml RNase A (Qiagen). Prior to final elution, spin columns were placed at 70 °C for 1 m whilst plates were incubated at RT for 5 m. Eluted DNA was stored at -20 °C until required.

2.6 Extraction of *S. uberis* total RNA

RNA was extracted from *S. uberis* cultures using the RNeasy Mini Kit (Qiagen). Briefly, a 2 ml overnight culture of the desired isolate was prepared by inoculation of the desired medium from a glycerol stock. After the required incubation period, 1 ml of the *S. uberis* culture was aliquoted and centrifuged at 17,900 × *g* for 5 m. The cell pellet was re-suspended in 100 μl of 3 mg/ml lysozyme prepared in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and incubated at RT for 15 m. To the samples, 350 μl buffer RLT containing 3.5 μl β-mercaptoethanol was added; these were mixed by vortexing and then passed 2 to 3 times though a 23 gauge needle to facilitate lysis. Cell debris was removed by centrifugation for 2 m; the supernatant was then mixed with 200 μl 100 % ethanol by pipetting up and down, and then loaded onto an RNeasy column. Columns were centrifuged for 15 s at 8,000 × *g* and flow-through was discarded. To each column, 700 μl Buffer RW1 was applied and centrifuged for 15 s as previously. Flow-through was again discarded and membrane washed twice with 500 μl Buffer RPE, centrifuging for 15 s on the first wash and 2 m on the second. The RNeasy spin column was transferred to a new 2 ml collection tube and centrifuged at full speed for a further 1 m to eliminate residual buffer carryover. The column was transferred to a 1.5 ml collection tube and eluted with 50 μl RNase-free water (Qiagen) by centrifuging for 1 m at 8,000 × *g*. RNA was stored at -20 °C for a maximum of 1 week prior to DNase treatment and reverse transcription (RT).

2.7 Complementary DNA (cDNA) preparation

Into a 1.5 ml micro-centrifuge tube, 12.5 μl total RNA was aliquoted. DNase treatment was conducted using DNase I, amplification grade (Invitrogen) by addition of 2.75 μl 10×

DNase I buffer and 12.5 μl (containing 12.5 U) DNase I to the RNA. DNase treatment was conducted at RT for 15 m and then the reaction was stopped by addition of 2.75 μl 25 mM EDTA and incubation at 65 °C for 10 m.

Two 10 μl aliquots of DNase-treated RNA were prepared and the following added to each aliquot; 0.3 μl (150 ng) random primers, 1 μl 10 mM nucleotide dNTP mix (10 mM each) and 8.7 μl RNase-free dH_20. Samples were heated to 65 °C for 5 m then incubated on ice for at least 1 m, prior to brief centrifugation to collect liquid at the bottom of the tubes. The remaining reagents (Invitrogen) were added to the aliquot designated as the test sample; 4 μl 5× First strand buffer, 1 μl 0.1M DTT, 1 μl RNase OUT and 1 μl SuperScript® III RT. To the second aliquot (the control replicate), all the above reagents were added except the SuperScript® III RT which was replaced with 1 μl RNase-free dH_20, to demonstrate that subsequent PCR products obtained were the result of cDNA, not gDNA, amplification. Samples were mixed by gently pipetting up and down then incubated at 25 °C for 5 m, 50 °C for 45 m and finally inactivation was conducted by heating samples at 70 °C for 15 m. To remove remaining RNA and reduce subsequent undesired RT by *taq* polymerase in negative samples, 2 μl of 1 mg/ml RNase A was added to each sample (both test and control replicates) and incubated at 37 °C for 30 m. The treated cDNA was stored at -20 °C until required.

2.8 Plasmid DNA extraction

2.8.1 Small-scale plasmid preparation

The QIAprep Spin Mini kit (Qiagen) was used for the small scale extraction of plasmid DNA from bacterial samples. A single colony was used to inoculate 5 ml medium (with antibiotics if required). After approx. 24 h this culture was centrifuged at 3,893 × *g* for 15 m to pellet cells. Cell pellets were re-suspended in 250 μl buffer P1 and transferred to 1.5 ml micro-centrifuge tubes. To prepare plasmids from *S. uberis*, at this stage 2 mg/ml lysozyme was also added and the re-suspended cells incubated at 37 °C for 30 m to promote cell lysis. This step was not required for plasmid preparation from *E. coli*. Re-suspended cells were mixed thoroughly with 250 μl buffer P2, followed by 350 μl of buffer N3. After further mixing, sample was centrifuged at 17,900 × *g* for 10 m. Supernatant was transferred to a QIAprep spin column and centrifuged for 1 m. Flow-through was discarded and membrane washed with 500 μl buffer PB, followed by 750 μl buffer PE, with flow-through being discarded after each wash. After the final wash, the

column was centrifuged for a further 1 m to remove all traces of wash buffer. DNA samples were eluted in 30 to 50 μl of RNase-free dH_20, with columns being incubated at RT for 1 m prior to final centrifugation.

2.8.2 Large-scale plasmid preparation

To prepare larger volumes of higher quality plasmid DNA (*i.e.* for transforming bacteria) the Qiagen plasmid maxi kit was used. A bacterial culture was prepared from glycerol stocks into 500 ml BHI broth; after overnight incubation, the culture was centrifuged at 6,000 × *g* for 15 m at 4 °C to harvest cells. The cell pellet was re-suspended in 10 ml buffer P1. As mentioned previously, when preparing plasmid DNA from *S. uberis*, 2 mg/ml lysozyme was also added to cell pellets and the sample incubated at 37 °C for 30 m prior to continuation with protocol. Re-suspended cells were mixed thoroughly with 10 ml buffer P2 and incubated at RT for 5 m prior to addition of 10 ml buffer B3. The sample was incubated on ice for 20 m prior to centrifugation at 20,000 × *g* for 30 m at 4 °C. Supernatant was filtered through a 1.0 μM syringe filter (Sartorius) then applied directly onto a pre-equilibrated (using 10 ml buffer QBT) Qiagen-tip 500 and allowed to enter the resin by gravity flow. Column was washed twice with 30 ml buffer QC and then DNA was eluted with 15 ml buffer QF. To precipitate DNA, 10.5 ml of RT isopropanol was added to the column eluate. After mixing, eluate was immediately centrifuged at 15,000 × *g* for 30 m. Supernatant was carefully removed, DNA pellet was washed with 5 ml 70 % ethanol (at RT) and then centrifuged at 15,000 × *g* for a further 10 m. The supernatant was decanted carefully and the remaining DNA pellet was air-dried for *ca.* 10 m prior to being re-dissolved in 100 μl RNase-free dH_20.

2.9 DNA electrophoresis

For visualisation of DNA, samples were electrophoresed through molecular biology-grade agarose gels. The electrophoresis apparatus employed was the Sub Cell or Mini Sub Cell GT system (Bio-Rad Laboratories Ltd., Hertfordshire, UK). Agarose gels containing between 1 and 2 % agarose (Promega, Madison, WI) were cast with 0.5x Tris-acetate-EDTA (TAE) buffer, prepared from 50× concentrate (Serva, Heidelberg, Germany), and 1× Gel Red (Biotium Inc., California, USA) to allow subsequent visualisation of DNA. DNA samples were mixed with 5 μl 6× loading dye and electrophoresed through the gel alongside a 1 kb or 100 bp DNA ladder using a current of between 50 to 120 V/cm provided by a PowerPac (Bio-Rad). The Alphamager™ 2200 (Alpha Innotech, California,

USA) was used to visualise DNA (over UV light) and images produced were printed and stored electronically as TIFF or JPEG files.

2.10 Polymerase chain reaction (PCR)

2.10.1 Primer Design

Oligonucleotide primers were produced to order by Sigma-Genosys Ltd. (Haverhill, Suffolk, UK) and are described in **Tables 2.4 to 2.6**. Lyophilised primers were reconstituted to 100 pmol/μl solutions using 5 mM Tris-HCl (pH 8.0). To produce working stock solutions, primers were further diluted in 5 mM Tris-HCl (pH 8.0) to a concentration of 10 pmol/μl. Primer stock solutions were stored at -20 ºC until required.

2.10.2 PCR using Qiagen *Taq* polymerase Master Mix

Polymerase Chain Reactions were conducted in 0.2 ml PCR tubes (Elkay Laboratory Products Ltd., Hampshire, UK) using either the GeneAmp® PCR system 9700 (Applied Biosystems, California, USA) or MBS Satellite Thermal Cycler 0.2G (Thermo Fisher Scientific, Leicestershire, UK). A typical PCR reaction contained 15.0 μl *Taq* Master Mix, 30 pmol forward and reverse primers and 6.0 μl RNase-free dH_20. The final concentration of $MgCl_2$ in a standard reaction when using the *Taq* Master Mix was 1.5 mM; on occasion, additional $MgCl_2$ was added to give a final concentration of $MgCl_2$ in the reaction mixture of between 1.5 mM and 1.8 mM (the volume of water added was also adjusted accordingly). Finally, 300 ng of template DNA was added to the mixture and mixed gently by pipetting.

The amplification protocol was always initiated with a single denaturation step of 94 ºC for 5 m and completed with an extension cycle of 72 ºC for 7 m. Between 30 and 40 amplification cycles were conducted between these steps, beginning with a denaturing step of 94 ºC and ending with an extension step of 72 ºC. The temperature for the annealing step between the denaturation and extension steps varied depending upon the primers used (**Tables 2.4 to 2.6**). Amplification steps were conducted for between 30 and 120 s based on the predicted size of the PCR product. Where PCR was used to amplify across large ligated PCR products, as in the targeted mutagenesis protocol (**Section 6.2.5.1**), the PCR mixture was increased to a total volume of 50 μl.

Table 2.4: Primer sequences utilised for characterisation of *S. uberis* isolates by MLST.

Primer	Sequence (5' – 3')	Target location	Reference	An[A]
Target: Carbamate kinase - Involved in the arginine dihydrolase pathway to produce ATP				
arcC F	GTT TGT GAC GCA AAA TCT TTA TCG ATA ACA	Acc. # AM946015 1311658-1311687	Coffey, 2006	56
arcC R	ACT CAT GGT AAC GGA CCA CAA GTT GGT AAC	Acc. # AM946015 1312146-1312175	Coffey, 2006	
Target: D-alanine-D-alanine ligase - Involved in cell-wall biosynthesis				
ddl F	GTC TAT ATT GAA GGT AAT GAC TTG GAA GAC TGT	Acc. # AM946015 1245800-1245832	Coffey, 2006	60
ddl R	TAC ATG GAC CACT GAG TGA ATC CAG GCA TAG TAT TC	Acc. # AM946015 1245330-1245365	Coffey, 2006	
Target: Glyceraldehyde-3-phosphate dehydrogenase - Role in glycolysis and gluconeogenesis as well as diverse non-glycolytic functions				
gapC F	TTG GTA TTA ACG GTT TCG GTC	See reference	Zadoks, 2005	50
gapC R	CAA GTT GAG CAG TGT AAG ACA TTT C	See reference	Zadoks, 2005	
Target: Glucokinase - Involved in the first step of glycolysis, catalysing phosphorylation of glucose				
gki F	GAC CGG ACC CAA AAC ACA GTC ACA GGT GCT TTT	Acc. # AM946015 1286411-1286443	Coffey, 2006	56
gki R	AAG AGA ATC TGG ATT TAG GAT ATT TGA AAT ATT	Acc. # AM946015 1285880-1285912	Coffey, 2006	
Target: Plasminogen activator A - Activates plasminogen to plasmin (which breaks down fibrin)				
pauA F	TTC ACT GCT GTT ACA TAA CTT TGT G	Acc. # AM946015 1772063-1772087	Zadoks, 2005	50
pauA R	CCT TTG AAA GTG ATG CTC GTG	Acc. # AM946015 1773017-1773037	Zadoks, 2005	
Target: Plasminogen activator B - Activates plasminogen to plasmin				
pauB F	CGG CTA GCT AGA ATA AGG GAG	Acc. # AJ314852 83-103	This study	57
pauB R	GAT ATT GAT TGA GGA TAC TTT AAG CGG	Acc. # AJ314852 1317-1343	This study	
Target: Flanking region around plasminogen activator locus				
ER45	GAG ATT CCT CTC TAG ATA TCA	See reference	Ward, 2002	50
ER46	GGG CTG CAG ATC CGT TAA AAA ATG ACA TTA ATA T	See reference	Ward, 2002	
Target: Transketolase 2 - Provides a link between the glycolytic and pentose-phosphate pathways				
recP F	AAT TCA GGT CAC CCT GGC TTA CCA ATG GGT GCA GCC	Acc.# AM946015 286564-286599	Coffey, 2006	60

recP R	TGT GAA AGC CAT TGA TGT TGG ACC ATC AAG TGA AAT	Acc.# AM946015 287059-287094	Coffey, 2006	
Target: Thymidine kinase - Catalyzes the ATP-dependent phosphorylation of thymidine				
tdk F	TAT TTT CAT TTC ATA ATA AGT TAG TGG ATT TAG TAA	Acc.# AM946015 847518-847553	Coffey, 2006	60
tdk R	TTG ATC ATA TAT ATT CAT GTT ATG AAT CGT TCT CCT	Acc.# AM946015 848275-848310	Coffey, 2006	
*tdk*TL F	TGA CTA TTG AAA CCG CTA TTA TG	Acc.# AM946015 847486-847508	This study	57
*tdk*TL R	AAT GTT TAC GAC AAA CTG GAA TG	Acc.# AM946015 848224-848246	This study	
Target: Triosephosphate isomerise - An enzyme involved in glycolytic pathway				
tpi F	GTT ATT GGT CAT TCA GAA CGT CGT GAT TAC TTC	Acc.# AM946015 572934-572966	Coffey, 2006	60
tpi R	GTC AAG TAA TGC TAA GAA GCT ATC TGC TTC AAG TGA	Acc.# AM946015 573369-573404	Coffey, 2006	
Target: Acetyl-CoA acetyltransferase 2 - Involved in the tri-carboxylic acid cycle				
yqiL F	TTT CTT CTT TGA AAC GAT TAT TTT TAA GTG CTT CAG	Acc.# AM946015 1392386-1392421	Coffey, 2006	56
yqiL R	CAA GCT CTA AGA ACA CCA ATT GGT GCA TTC GGA GGA	Acc.# AM946015 1391848-1391883	Coffey, 2006	
Target: Acetyl-CoA acetyltransferase - Involved in the tri-carboxylic acid cycle				
Acetyl F	GCC ATTATT TCA AGG CCT TTT TCT TTA GC	Acc. # AM946015 1795467-1795495	This study	51
Acetyl R	GGT AAT ATT ATT GCC ATT AAT AGT GG	Acc. # AM946015 1796082-1796107	This study	
Target: 16S ribosomal RNA - Protein manufacture				
16 S *uberis* F	CGC ATG ACA ATA GGG TAC A	Acc.# AM946015 Multiple sites	Hassan, 2001	58
16 S *uberis* R	GCC TTT AAC TTC AGA CTT ATC A	Acc.# AM946015 Multiple sites	Hassan, 2001	

[A] Annealing temperature (An) in ^{o}C utilised during PCR for amplification of, in most cases, an internal region of approx. 500 bp.

Table 2.5: Primer sequences utilised for the amplification and characterisation of genes from *S. uberis* isolates with homology to *S. epidermidis* biofilm associated genes.

Primer	Sequence (5' – 3')	Target	Reference	An[A]
***icaA* homologue. *S. uberis* ID:** Hyaluronan synthase (*hasA*) - Hyaluronic acid capsule production				
hasA gene F	GGG ATT ATT CTA TTA ACC	Acc.# AM946015 1677961-1677978	This study	44
hasA gene R	GAG ATT AAA TTC TTG AGC C	Acc.# AM946015 1677040-1677058	This study	
***icaB* homologue. *S. uberis* ID:** Polysaccharide deacetylase (SUB 0809) – Function unknown				
SUB 0809 gene F	CCC CTT TTT CTA ATC CTG	Acc.# AM946015 789686-789703	This study	48
SUB 0809 gene R	GCG ATA ACA GGA TTT CGG	Acc.# AM946015 788833-788850	This study	
***icaC* homologue. *S. uberis* ID:** Membrane protein (SUB 1487) – Function unknown				
SUB 1487 gene F	GGA TTA TCC AGG TTT TAG TTT ATG G	Acc.# AM946015 1480261-1480285	This study	49
SUB 1487 gene R	CGT ATT GAC GAA TGA TTC AGC AAA GCC	Acc.# AM946015 1479534-1479560	This study	
***icaD* homologue. *S. uberis* ID:** Membrane protein (SUB 0701) – Function unknown				
SUB 0701 gene F	GGT ATT ATT CTT ACT CTA TTA CC	Acc.# AM946015 678118-678140	This study	43.5
SUB 0701 gene R	CCG CAG GTT CAC CTT GAG ACC AAG GC	Acc.# AM946015 679587-679612	This study	
***luxS* homologue. *S. uberis ID*:** S-ribosylhomocysteinase (*luxS*) – Potential role in quorum sensing				
luxS gene F	GGT GTC CTC TTC TCA TAA C	Acc.# AM946015 1396545-1396563	This study	46
luxS gene R	CAA GAT GTC GTT CAA AAG C	Acc.# AM946015 1397128-1397146	This study	

[A] Annealing temperature (An) in ^{o}C required for PCR.

Table 2.6: Primer sequences utilised for *S. uberis* mutagenesis protocols.

Primer	Sequence (5' – 3')	Target	An[D]
Polysaccharide deacetylase (*icaB* homologue) gene replacement			
Amplification of 1 kb region upstream of gene			
SUB 0809 01	GGG AAA CTT CTG CTG AAA TG	Acc.# AM946015 790704-790723	55
SUB 0809 02	*GCG CGC* gga tcc CCG GCG CAT GTT AGT TAC [A, B]	Acc.# AM946015 789731-789748	
Amplification of 1 kb region downstream of gene			
SUB 0809 03	*GCG CGC* gga tcc ATA CCA TAA GAA AAT GTC TTC [A, B]	Acc.# AM946015 788804-788824	45
SUB 0809 04	GTC TAT TGC CAA GTT G	Acc.# AM946015 787835-787850	
Amplification across ligated PCR constructs to produce deletion product for targeted replacement of polysaccharide deacetylase gene (primers SUB 0809 01 SUB 0809 04)			45
Amplification of region upstream of gene to demonstrate loci conservation between isolates			
SUB 0809 11	GCT GTA ACA ACA TGT TTT CC	Acc.# AM946015 790219-790238	41
SUB 0809 12	GGC CTG ATT AGT GG	Acc.# AM946015 789551-789564	
Amplification of region downstream of gene to demonstrate loci conservation between isolates			
SUB 0809 13	CGA TAG CTA TCG CC	Acc.# AM946015 788999-789012	49
SUB 0809 14b	GGG TCC TTC AAG AAA CC	Acc.# AM946015 788301-788317	
Amplification of internal region of gene for RT-PCR			
SUB 0809 21	CAG AGT TCT CAC TCA GAA TG	Acc.# AM946015 789319-789338	50
SUB 0809 22	GGT TAA GGT AAA CCA TGG AAT C	Acc.# AM946015 789031-789052	
Amplification across gene for sequencing and demonstration of deletion			
SUB 0809 31	GCT ATT TTT AGT GAC ATA CAT TTC TCC	Acc.# AM946015 789918-789944	48
SUB 0809 32	GGA ACT CTT TGA TTT TGT TGA GTA CGG	Acc.# AM946015 788695-788721	
Primers out-with 1 kb upstream/downstream region to demonstrate deletion is within chromosome not plasmid			
SUB 0809 Flank 01	CCA TAA CTG ATG TTG CTG TTG G	Acc.# AM946015 790843-790864	50
SUB 0809 Flank 02	GGT TAT GTT GCA GAT AAC ATG ATG TCT GG	Acc.# AM946015 787628-787656	
Hyaluronan synthase (*icaA* homologue) gene replacement			
Amplification of 1 kb region upstream of gene			
hasA 01	GGT CTT TTA GAG GCT TTG GTG G	Acc.# AM946015 1679068-1679089	60
hasA 02	*GCG CGC* ggt acc GAG ATT TTT TAG TTT TTC CAT AAT TCC [A, K]	Acc.# AM946015 1678069-1678095	

Amplification of 1 kb region downstream of gene			
hasA 03	*GCG CGC* ggt acc CGT AAA AAG ACA AGT AAA TAA TTC [A, K]	Acc.# AM946015 1676833-1676856	55
hasA 04	CAT GAT AAG GCG GTA AAT ACC	Acc.# AM946015 1675849-1675869	
Amplification across ligated PCR constructs to produce deletion product for targeted replacement of hyaluron synthase gene (Primers *hasA* 01 & 04)			50
Amplification of internal region of gene for RT-PCR			
hasA 21	CGT GCT GCT CAG TCT GTT ACG GG	Acc.# AM946015 1677431-1677453	51
hasA 22	GGG ACA CCA AGA AAT GTT TGT GAG G	Acc.# AM946015 1677332-1677356	
Amplification across gene for sequencing and demonstration of deletion			
hasA 31	GCT TTA ACA TAT CAG ATT TAC AAA AAT GAT GG	Acc.# AM946015 1678142-1678173	52
hasA 32	GGA TTT TTC TGT GCT AAT AAT ACA CTT AAT GAT AGG	Acc.# AM946015 1676741-1676776	
Amplification out-with 1 kb upstream or downstream region to demonstrate deletion is within chromosome not plasmid			
hasA Flank 01	GCT AGA TTT AAT TGC TAT CCG	Acc.# AM946015 1679379-1679399	50
hasA Flank 02	GGT AAA TCA AAA TCT TCA TTC ATC ATT GG	Acc.# AM946015 1675730-1675758	
General			
Amplification within cloning site of pG$^+$host9			
pGh9 01	CCA GTG AGC GCG CGT AAT ACG	See reference [C]	54
pGh9 02	GGT ATA CTA CTG ACA GCT TCC	See reference [C]	
Positive control for RT-PCR			
recA F	GGT TAT CGA CTC TGT TGC GGC	Acc.# AM946015 1764869-1764889	51
recA R	GGA TTT CCA AAC ATA ACA CC	Acc.# AM946015 1764695-1764714	

[A] Tail sequences are italicised.

[B] Endonuclease recognition sites for *Bam*HI are indicated by lower case letters.

[C] Reference, Fontaine et al. 2004. All remaining primers were designed during this study.

[D] Annealing temperature (An) in ^oC required for PCR.

[K] Endonuclease recognition site for *Kpn*I are indicated by lower case letters.

2.10.3 Colony PCR

For routine screening purposes, PCR was conducted directly from bacterial colonies. Colonies of interest and the appropriate controls were suspended in 100 μl of InstaGene Matrix (Bio-Rad) using a sterile toothpick. Samples were boiled at 100 °C for 3 m, vortexed briefly and centrifuged at 17,900 × *g* for 3 m to pellet the cell debris. Supernatants were aliquoted into clean micro-centrifuge tubes for use in PCR. Reaction mixtures for colony PCR generally consisted of 24 μl *Taq* Master Mix, 18.4 μl RNase-free dH_20, 1.6 μl of 25 mM $MgCl_2$ (to give a final concentration of $MgCl_2$ in the reaction mixture of 2.3 mM, including $MgCl_2$ that which was already present in the Master Mix and that which was added separately), 50 pmol fwd and rev primers and 5.0 μl template DNA.

2.10.4 Random Amplified Polymorphic DNA (RAPD) Typing

Typing was conducted using Ready-To-Go RAPD Analysis beads (GE Healthcare, Bucks, UK) following the manufacturer's protocol. Briefly, to a tube containing a RAPD bead 25 pmol of RAPD primer 5 (5'-AACGCGCAAC-3') and 50 ng gDNA were added, with the solution being made up to 25 μl with RNase-free dH_20. The tube contents were mixed by gently vortexing and then collected at the bottom of the tube by brief centrifugation. The samples were then subjected to the following amplification conditions: 1 cycle of 95 °C for 5 m, 45 cycles of 95 °C for 1 m, 36 °C for 1 m and 72 °C for 2 m, followed by a final single cycle of 72 °C for 10 m. The PCR products were analysed through a 20 cm long, 2 % (w/v) agarose gel against 1 kb and 100 bp DNA ladders. Gel images were saved as TIFF files and imported into BioNumerics v.6.1 software (Applied Maths NV, Belgium) for analysis. Isolate identification was assigned to the appropriate lanes and bands were automatically identified by the computer; where appropriate, additional bands were highlighted manually. Banding patterns for completed isolates were then compared to all other isolate using the default settings of the programme permitting the production of a phylogenetic tree.

2.10.5 Reverse transcription PCR

Reverse transcription PCR was conducted using cDNA (prepared as described in **Section 2.7**). A typical reaction mixture contained 1 U Platinum® *Taq* Polymerase (Invitrogen), 1× reaction buffer, 1.5 mM $MgCl_2$ (final concentration), 0.2 μM fwd and reverse primers, 0.2 mM each of dATP, dTTP, dCTP and dGTP and 1.0 μl cDNA from RT positive and negative samples (made up to 30 μl in RNase-free dH_20). Conditions for PCR using cDNA

were the same as those described previously for gDNA, with between 30 and 35 amplification cycles being used. Successful RNA extraction without contaminating gDNA resulted in a band of the predicted size being amplified from the RT positive sample, whilst no band was produced from the RT negative sample.

2.11 DNA Purification

2.11.1 Purification of PCR products

PCR products were generally purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's guidelines. To one volume of PCR product, five volumes of buffer PB was added, mixed gently, and the solution applied to a QIAquick spin column. Column was centrifuged at 17,900 × *g* for 1 m and flow-through discarded. The membrane was washed with 750 μl buffer PE, the flow-through discarded and the column spun again, to ensure all traces of wash buffer were removed. Finally, DNA was eluted into a clean 1.5 ml micro-centrifuge tube using 30 μl RNase-free dH_20. Prior to final centrifugation, column was left to stand at RT for 1 m to improve DNA elution.

On occasion, large-scale PCR purification was also required, for example during MLST studies when PCR was conducted in 96-well plates to increase throughput. On these occasions purification was conducted using the MinElute™ 96 UF PCR Purification kit (Qiagen) and vacuum manifold protocol. Purification was conducted according to the manufacturer's guidelines. Briefly, PCR products were applied to the MinElute™ plate using a multichannel pipette. The plate was placed into the vacuum manifold and vacuum applied until all liquid had been drawn through the plate. The plate was then removed from the manifold and tapped gently onto clean paper towels to remove any remaining flow-through. To each well of the plate 30 μl of RNase-free dH_20 was applied and pipetted up and down approx. 20 times to elute DNA; this being subsequently transferred to a fresh 96-well PCR plate for storage. Purified DNA was stored at -20 °C until required.

2.11.2 Purification of DNA from agarose

DNA electrophoresed through agarose gels was purified using the QIAquick Gel extraction kit (Qiagen) following the manufacturer's provided protocol. The required band was visualised over a UV light box (Hybaid Crosslinker, Hybaid Ltd. Middlesex, UK), and using a clean scalpel, was excised from the gel and transferred to a clean micro-

centrifuge tube. The gel slice was weighed and three volumes of buffer QG added for each volume of gel (100 mg equivalent to 100 μl). The agarose was solubilised by incubation at 50 °C and then one volume of RT isopropanol was added to the solution which was then mixed gently before being applied to a QIAquick column. Centrifugation at 17,900 × *g* for 1 m bound DNA to the membrane and resulting flow-through was discarded. To remove all traces of agarose, 500 μl of buffer QG was applied to the column. After further centrifugation, the membrane was washed with 750 μl buffer PE, the flow-through discarded and the column spun again to remove all traces of wash buffer. To elute DNA, 30 to 50 μl RNase-free dH_20 was applied to the column, and to improve DNA yield, columns were incubated at RT for 1 m prior to final centrifugation. Purified DNA was stored at -20 °C until required.

2.12 DNA/RNA Quantification

Nucleic acids (DNA and RNA) were quantified, and the purity determined using the NanoDrop® ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Inc., Delaware, USA). A 1.5 μl aliquot of each sample was applied directly onto the analyser with no requirement for pre-dilution. Highly-purified DNA had absorbance values at 260nm/280nm and 260nm/230nm of greater than 1.8, whilst with RNA, values greater than 2 were expected.

2.13 DNA Sequencing

2.13.1 Sequencing of PCR products

Automated sequencing of PCR products prepared in 96-well plates was conducted by staff at the Genetics Core at the Wellcome Trust Clinical Research Facility, based at the Western General Hospital (Edinburgh, UK). All additional sequencing was conducted by Eurofins MWG Operon (Ebersberg, Germany) or Cogenics (Essex, UK). DNA was always sequenced from both the forward and reverse strands to increase confidence in data obtained. Sequence data were received as BioEdit, or Word files and forward and reverse sequences were aligned and edited using Clone Manager (**see Section 2.24**).

2.13.2 Whole genome sequencing

Genomic DNA for whole genome sequencing was prepared as described in **Section 2.5** with the only amendment to the protocol being that DNA was eluted in 60 μl TE buffer containing 20 mg/ml RNase A. Genomic DNA (10 μg) from each isolate was supplied to 'the GenePool' Next Generation Sequencing and Bioinformatics facility at the University of Edinburgh where sequencing was conducted using Illumina SOLEXA apparatus and indexed, paired-end parameters which generated read pair lengths of approx. 50 bp with approx. 40-fold coverage of each genome.

2.14 General DNA manipulation

2.14.1 Restriction endonuclease digestion

The required restriction endonucleases (Promega) were used to digest DNA following the protocol recommended the manufacturer. Typically, a 10 μl reaction contained, 10 U enzyme, 1× reaction buffer, 10 μg acetylated bovine serum albumin (BSA) and between 1 and 5 μl DNA. The reaction was made up to the final volume with RNase-free dH_20. Incubation was routinely conducted at the temperature recommended by the manufacturer for 2 h, occasionally, however, incubation needed to be conducted overnight. To minimise star activity, the recommended buffer was used, the final concentration of glycerol (from the enzyme storage buffer) in the reaction was limited to ≤10 % and the reaction tubes were sealed with parafilm to minimise evaporation. Digested DNA was analysed by electrophoresis. Clone Manager was used to predict the fragment sizes of products from digestion reactions, based on the analysis of DNA sequence data.

2.14.2 DNA ligation

Quick ligation of PCR products was conducted using quick DNA ligase (New England Biolabs). For this protocol, 5 μl of each purified and digested PCR product was mixed with 1 μl Quick ligase and 10 μl Quick ligase buffer. Ligation reactions were conducted at RT for just 5 m with no heat inactivation being required. Ligation of DNA into a prepared vector was conducted using T4 DNA ligase (Promega). In this reaction, 6 U T4 DNA ligase, 1× T4 DNA ligase buffer and 2 μl 10 mM rATP were mixed with insert and vector DNA, typically 8 or 9 μl insert was added to 1 or 2 μl vector. Ligation was incubated at 4 °C for at least 24 h.

2.14.3 Dialysis of ligated DNA and plasmid DNA

Using tweezers, a 0.025 μM VS filter (Millipore, Hertfordshire, UK) was pre-wet by being carefully placed (shiny side up) into a cell culture dish containing dH_20 for approx. 5 m. The DNA sample was then gently pipetted onto the top centre of the filter. Dialysis was conducted for approx. 10 m at RT after which the sample was carefully recovered from the surface of the filter using a pipette.

2.14.4 PCR Polishing

Where blunt-ended DNA inserts were required, DNA was polished using the Stratagene PCR polishing kit (Agilent Technologies, California, USA). In a sterile 0.5 ml micro-centrifuge tube the following reagents were added in order: 5.0 μl insert DNA, 1× *Pfu* DNA polymerase buffer, 1.0 μl 10 mM dNTP mix, 2.5 U cloned *Pfu* DNA polymerase and 2 μl RNase-free dH_20. Components were mixed gently, covered with a layer of mineral oil and incubated at 72 °C for 30 m. The reaction was placed on ice after the required incubation period and used directly for ligation into the appropriate vector.

2.14.5 Cloning into PCR® II-Blunt-TOPO®

The Zero Blunt® TOPO® PCR Cloning kit (Invitrogen) was used according to the manufacturer's protocol. Briefly, in a 0.2 ml micro-centrifuge tube the following reagents were added in order: 3.5 μl polished DNA insert, 1 μl salt solution, 0.5 μl RNase-free dH_20 and 1 μl PCR® II-Blunt-TOPO®. Reagents were mixed gently and incubated at RT for 15 m. Reactions were placed on ice and immediately used to transform One Shot® chemically competent cells (Invitrogen).

2.15 Preparation of electro-competent cells

2.15.1 Preparation of electro-competent *S. uberis* cells

A 5 ml overnight *S. uberis* culture was used to inoculate 95 ml of pre-warmed BHI broth containing 0.5 % yeast extract and 50 μg/ml hyaluronidase (with or without between 1 and 3 % glycine). The culture was incubated at 37 °C until the $OD_{560\ nm}$ was between 0.2 and 0.4. The culture was then transferred to two chilled 50 ml BD Falcon™ tubes (Becton, Dickinson U.K. Ltd., Oxford, U.K.) and centrifuged in a pre-chilled rotor at 3,893 × *g* for 30 m at 4 °C to pellet the cells. Supernatant was discarded and pellets re-suspended in 50 ml of ice cold 15 % (v/v) glycerol. Re-suspended cells were centrifuged

again under the same conditions to wash the cells. Cell pellets from both tubes were re-suspended and pooled into a single tube in a total volume of 25 ml of cold 15 % glycerol and then centrifuged once further. The remaining pellet was re-suspended into 500 μl of 15 % glycerol. Aliquots of the competent cells (75 μl) were prepared in chilled 0.5 ml tubes and either used immediately for electroporation or flash-frozen in a dry ice/ethanol water bath and stored at – 70 °C until required.

2.15.2 Preparation of electro-competent *E. coli* cells

Competent *E. coli* cells were prepared, utilising an established protocol (Froehlich and Scott, 1991). Briefly, 495 ml of pre-warmed LB broth was inoculated with a 5 ml overnight *E. coli* culture and incubated at 200 rpm at 37 °C until the $OD_{600\ nm}$ value was between 0.5 and 0.8. The culture was chilled in an ice water bath for *ca.* 30 m then transferred to two chilled 250 ml centrifuge pots and centrifuged in a pre-chilled rotor at 3,893 × *g* for approx. 30 m to pellet cells. Cell pellets were gently re-suspended in 250 ml sterile ice-cold water and then centrifuged as previously. This was repeated, the supernatant discarded and cell pellet re-suspended in 20 ml of ice-cold sterile 10 % glycerol. Re-suspended cells were transferred to a pre-chilled 50 ml tube and centrifuged for 30 m to pellet the cells again. Cells were finally re-suspended in 800 μl of 10 % glycerol. Aliquots of 50 μl were transferred to 0.5 ml micro-centrifuge tubes and either used immediately or stored at -70 °C following flash freezing in an ethanol/dry ice water bath.

2.16 Transformation of bacterial cells

2.16.1 Quick transformation protocol (*E. coli*)

Colonies were scraped from agar plates into 100 μl of cold transformation solution (0.1 M $CaCl_2$, 0.1 % PEG 8000) and incubated on ice for 5 m. Plasmid DNA (1 μl) was added to the cell suspension and incubated on ice for 8 m. The suspension was then incubated at 42 °C for 40 s and then returned onto ice for 2 m. Cells were recovered in 900 μl of SOC medium without Mg^{2+} salts (**Appendix 1**), transferred to a 14 ml tube and incubated at 37 °C for 1 h at approx. 200 rpm. For identification of transformants, aliquots of 50, 100 and 200 μl were spread onto LB agar plates containing between 300 and 400 μg/ml of ampicillin and incubated at 37 °C overnight.

2.16.2 Transformation of competent cells

2.16.2.1 Transformation of One Shot® chemically competent cells

A single vial of One Shot® chemically competent cells (Invitrogen) was thawed on ice. Once defrosted, 2 μl PCR® II-Blunt–TOPO® construct was added to the cells and mixed gently. Cells and construct were incubated on ice for 15 m, heat shocked by incubation at 42 °C for 30 s, then immediately returned to incubation on ice. Cells were recovered by addition of 250 μl SOC medium (Invitrogen) and incubation at 37 °C × 200 rpm for 1 h. Aliquots (50 and 200 μl) were spread onto pre-warmed LB plates with 50 μg/ml kanamycin and incubated at 37 °C overnight.

2.16.2.2 Transformation of *S. uberis* electro-competent cells

Between 1 and 5 μl of plasmid DNA was added, on ice, to 75 μl competent cells along with 5 μg of TypeOne™ Restriction Inhibitor (Epicentre® Biotechnologies, Wisconsin, USA) where required. Transformation was conducted at 25 μF, 2.4 KV and 100 Ω or 25 μF, 1.25 KV and 600 Ω in a pre-chilled 0.1 cm electrode gap cuvette, with time constants of approx. 2.5 ms typically being obtained. Following electroporation, cells were recovered immediately into 1 or 10 ml pre-warmed BHI broth containing 0.5 % yeast extract and incubated at the desired temperature for approx. 2 h. Subsequently, transformed cells were identified following plating of transformation reactions onto BHI agar containing 1 or 5 μg/ml erythromycin. Cells from transformation reactions were also serially-diluted and 3 × 20 μl aliquots were plated onto BHI agar, without antibiotics, to allow determination of transformation efficiencies. Plates were incubated at 28 or 37 °C as appropriate until colony growth became evident.

2.16.2.3 Transformation of *E. coli* electro-competent cells

Competent cell aliquots were thawed on ice, or used directly following preparation, and mixed with 1 to 5 μl of the appropriate vector, then transferred to a pre-chilled 0.1 cm electrode gap cuvette. Electroporation was conducted at 200 Ω, 25 μF and 1.25 kV after which cells were recovered immediately in 1 ml LB (containing 2.5 mg $MgCl_2$, 2.8 mg $MgSO_4$ and 3.6 mg glucose). Cultures were incubated at 37 °C for 1 h prior to dilution and plating onto LB plates with erythromycin.

2.17 Allele replacement mutagenesis

Construction of targeted allele-replacement mutants was conducted, essentially as described elsewhere (Degnan *et al.*, 2000) utilising the temperature-sensitive pG$^+$host9 plasmid in a two-step recombination process. In the first instance, a preliminary integration of the pG$^+$host9-based constructs (described in **Chapter 6**), into the *S. uberis* chromosome by homologous recombination was performed. Subsequently, these so-called 'single cross-over intermediate' or 'co-integrant' mutants were used in a second round of recombination in which the plasmid excised from the chromosome and was cured by incubation of cultures at elevated temperature. These 'secondary cross-over' mutants were selected by virtue of their having lost (plasmid-encoded) antibiotic resistance and were then subjected to additional screening.

2.17.1 Selection of single cross-over intermediates

Medium containing antibiotics was inoculated using glycerol stocks of *S. uberis* 0140J transformed with the desired plasmid construct. Cultures were incubated at 28 ^{o}C for approx. 24 h, then diluted 50-fold in 5 ml of fresh, pre-warmed medium. Cultures were incubated at 28 ^{o}C for approx. 4 h until mid-log phase of growth; once the $OD_{600\ nm}$ was between 0.3 and 0.4, the temperature of incubation was shifted to 37 ^{o}C for a further 6 to 9 replication generations (approx. 4 h). After incubation, serial dilutions were prepared and 100 μl aliquots spread onto BHI agar containing 5 μg/ml erythromycin. Plates were incubated overnight at 37 ^{o}C permitting growth only of cells in which single-cross-over plasmid integration into the bacterial chromosome had occurred. Total viable counts were made to allow determination of integration frequency by also plating dilutions onto BHI agar with no antibiotics and incubating at 28 ^{o}C overnight.

2.17.2 Selection of double cross-over mutants

A single putative co-integrant colony (single cross-over mutant), prepared as described in **Section 2.17.1**, was used to inoculate 2 ml BHI broth containing 5 μg/ml erythromycin; and cultures were incubated at 37 ^{o}C for approx. 24 h. Glycerol stocks were prepared at this stage and were used to inoculate 10 ml BHI broth (no antibiotics) which was subsequently incubated at 28 ^{o}C for at least 20 h. The overnight cultures were diluted 100-fold into 10 ml pre-warmed BHI broth containing 5 μg/ml erythromycin, and propagated at 37 ^{o}C until mid-log phase of growth; during this time the integrated plasmids were excised from the chromosome. A novel ampicillin enrichment process was then employed (Walker *et al*. 2010, submitted for publication). The cultures were supplemented with erythromycin, such that unwanted cells retaining

the plasmid were expressing the erythromycin gene, the cultures were then further supplemented with ampicillin to a final concentration of 100 μg/ml. Actively-growing erythromycin-resistant cells were effectively killed by addition of ampicillin, leaving the cells which had lost the plasmid unharmed (due to the bacteriostatic effect of erythromycin). Growth rate was determined by $OD_{600\ nm}$ measurements; once growth had dropped to a static point, it was assumed that the ampicillin had exerted a maximal effect. It should be noted, however, that some erythromycin-resistant cells remained at this stage, meaning that cultures were 'enriched' for the desired mutants. At this stage, cells were harvested by centrifugation at 3,893 × *g* for 10 m, washed in 5 ml PBS to remove residual ampicillin, re-suspended in 5 ml PBS and serial dilutions were prepared and plated onto BHI agar without antibiotics. After incubation at 37 °C to allow colony growth, 100 to 200 single colonies were replica-plated onto BHI agar either free of, or containing 5 μg/ml erythromycin. Colonies unable to develop on erythromycin-containing plates were considered to have undergone excision and subsequent loss of plasmid. To confirm erythromycin sensitivity, these colonies were re-streaked onto selective and non-selective plates and incubated at 37 °C overnight. Glycerol stocks of these erythromycin sensitive colonies were prepared and these putative mutants were subjected to further analysis to demonstrate successful allele replacement.

2.18 Generation of an *S. uberis* mutant library

To prepare an *S. uberis* IS*S1* random insertion mutant library, a colony of *S. uberis* strain 0140J which had been transformed with the pGh9:IS*S1* plasmid was suspended in 5 ml BHI broth containing 0.5 % yeast extract and 5 μg/ml erythromycin. The culture was incubated at 28 °C for approx. 24 h. The overnight culture was diluted 100-fold into 20 ml BHI broth containing 0.5 % yeast extract, but no antibiotics, and was incubated at 28 °C until early log phase ($OD_{600\ nm}$ of approx. 0.2). The culture was then incubated at 37 °C for a further 3 h to reduce the copy number of the temperature-sensitive plasmid in the total population. Serial dilutions of this culture were prepared and used to inoculate BHI agar plates, which were incubated at 37 °C. The same dilutions were also plated onto two BHI agar plates containing 5 μg/ml erythromycin, one of which was incubated at 28 °C and the other at 37 °C. Colonies appearing at 37 °C with erythromycin were considered to have undergone successful integration of the pGh9:IS*S1* plasmid into the chromosome. The remaining culture was used to prepare glycerol stocks of the mutant library for storage at -70 °C prior to subsequent screening.

2.19 Whole cell mass spectrometry - BioTyping

Isolates of *S. uberis* were streaked for single colonies from glycerol stocks onto BHI agar plates. Plates were incubated at 37 °C for 24 to 48 h until large well defined colonies were visible. Colonies were suspended in the appropriate medium, using 10 colonies per 100 µl medium, and cells were prepared for mass spectrometry (MS). Individual protocols for cell preparation are shown in **Table 2.7**. Samples were vortexed for approx. 15 m and provided to staff of the Moredun Proteomics Facility (Moredun Research Institute, http://www.mri.sari.ac.uk/fgu-functional-genomics-services.asp) where samples were analysed using an Ultraflex II mass spectrometer (Bruker Daltonics, Bremen, Germany). A 1 µl aliquot of each sample was mixed with 1 µl of α-cyano-4-hydroxycinnamic acid (CHCA) matrix and spotted onto a 384 place aluminium target plate where they were allowed to dry. Spectra were acquired in linear, positive mode over a mass range of 2,000 to 20,000 Daltons. The accelerating voltage was 25 kV and the laser frequency was 35 to 45 Hz. For most samples, 4 biological replicates were conducted and 4 technical replicates were spotted from each onto a target plate, giving 20 spots per sample. From 10 different sites on each spot, 100 shots were collected and accumulated to give 1,000 shots for each spot. The instrument was externally calibrated using ProtMix 1 protein standards (Bruker Daltonics).

Raw data files were analysed using either the FlexAnalysis or BioTyping software (Bruker Daltonics). FlexAnalysis was used to visually compare mass spectra from different experiments. Smoothing and baseline subtraction was conducted on all spectra and where required the produced figures were exported and stored electronically. BioTyping analysis was used to further compare mass spectra using computer software incorporating various algorithms for principal component analysis and quantum clustering. This is discussed further in **Chapter 4**.

2.20 Biofilm assays

2.20.1 Congo red agar plate assay

To determine the production of biofilm-associated slime by test bacteria, a Congo red agar (CRA) plate assay was performed. The CRA was prepared by dissolving 18.5 g BHI powder, 25.0 g sucrose and 5.0 g Agar No.1 in 400 ml of dH_20. The medium was made up to a final volume of 500 ml with dH_20. Separately, in 125 ml dH_20, 1 g of Congo red was

Table 2.7: Protocols for chemical and physical methods used to prepare *S. uberis* isolates for MS.

Reaction mixture	Protocol
PHYSICAL	
Ribolysis (Physically disrupts cells by beating with beads) – Reference: Mandrell *et al.*, 2005	
100 μl dH_20 and 100 μl washed zirconium beads	Ribolysed using three 20 s bursts on max setting (6.5) of ribolyser (Hybaid). Samples incubated on ice for 1 m between bursts
Heat Treatment (Bursts cells, killing bacteria) – Reference: Williams *et al.*, 2003	
100 μl dH_20 or 100 μl dH_20 and 1× protease inhibitors	Incubated at 100 °C for 10 m to burst cells
CHEMICAL	
Acetonitrile (Dissolves proteins permitting ionisation) – References: Claydon *et al.*, 1996; Haag *et al.*, 1998	
100 μl 50 % acetonitrile (in 0.5 % TFA*) or 100 μl 50 % acetonitrile and 10mg/ml lysozyme	None
Ethanol (Lipid membrane solvent, disrupts normal molecular interactions and induces cell lysis) – References: Madonna *et al.*, 2000; Williams *et al.*, 2003	
100 μl 70 % ethanol	None
Lysozyme (Hydrolyses between peptidoglycan disaccharide subunits, cleaving the cell wall) - Reference: Smole *et al.*, 2002	
100 μl 10 mg/ml lysozyme or 100 μl 2 mg/ml lysozyme (in dH_20)	Incubated at 37 °C for 30 m
Hyaluronidase treatment (Catalyses the hydrolysis of hyaluronic acid capsule increasing capsule permeability)	
100 μl of 50 μg/ml hyaluronidase	Incubated at 37 °C for 2 h and then centrifuged to pellet cells. Pellets were washed twice in 100 μl dH_20, then re-suspended in the appropriate media, for example 100 μl acetonitrile

*TFA = Tri-fluoroacetic acid

dissolved to obtain an 8 g/l solution. Both solutions were autoclaved; medium and stain were cooled to 56 °C and Congo red solution was added to the medium to give a final concentration of 0.8 g/l (10 ml Congo red per 100 ml of medium). If required, erythromycin was also added to the cooled medium. Plates were poured and either used immediately or stored at 4 °C for no more than 1 month prior to use.

2.20.2 Microtitre plate biofilm assay

An overnight culture of the bacterial isolate for analysis (cultured in BHI broth) was diluted 100-fold into the media of interest. The inoculated media was aliquoted into the required wells of a flat-bottomed microtitre plate, with un-inoculated media used as controls. Plates were incubated statically at 37 °C for the required period. After incubation, growth medium containing planktonic cells was discarded and wells were washed 3 times with dH_20 or PBS to remove cells not adhering to plates. Plates were air dried for 45 m after which adherent cells were stained using 1 % methyl violet solution (Fisher Scientific, Leicestershire, UK). Plates were incubated for a further 45 m, after which, the stain was discarded. The wells were washed thoroughly with water until all excess stain was removed, and plates were again air dried for 45 m. Finally, 200 μl of 95 % ethanol was added to each well and incubated for 15 m. All incubations were conducted at RT. The $OD_{562\ nm}$ was measured to quantify the extent of biofilm formation, and plates were photographed to allow visual comparisons to be made.

2.20.3 Visualisation of biofilms using microscopy

For visualisation of *S. uberis* biofilms, overnight cultures of the required bacterial isolates (cultured in BHI broth) were prepared and diluted 100-fold into the test medium. Aliquots of the inoculated medium (1 ml) were transferred to the appropriate wells of a 24-well microtitre plate. Round cover-slips were placed into the wells and carefully submerged into each medium using a sterile pipette tip. The plate was incubated statically at 37 °C for the required period. Cultures were then discarded from the wells and cover-slips were washed gently in the well three times with PBS or dH_20 to remove unattached cells. After air drying for approx. 45 m, wells were stained with 1 % methyl violet (Fisher Scientific) for 45 m. After incubation, cover-slips were de-stained in the wells by rinsing thoroughly with water. While still wet, cover-slips were carefully lifted from the wells using tweezers and placed face up onto a paper towel to dry. Once dried, the cover-slips were fixed face down (biofilm side) onto a microscope slide using crystal mount™ (Biomeda Corp., California, USA). The slide was then examined using the

oil immersion lens of a Leica DM2000 light microscope (Leica Microsystems Ltd., Buckinghamshire, UK). Images were captured using a digital camera.

2.21 Siderophore assays

The siderophore assays utilised in this study were adapted from a well established and well described method which exploits the high affinity of siderophores for iron (Schwyn and Neilands, 1987). This method utilises chrome azurol S/ iron (iii)/ hexadecyltrimethyl ammonium bromide (HDTMA), a blue complex which changes to pink/orange when a strong chelator strips the iron from the dye. The method is applicable to the analysis of siderophores secreted in culture supernatants or from bacterial colonies on agar plates.

2.21.1 Chrome azurol S (CAS) agar plate assay

Siderophore detection was achieved using a CAS overlay assay described previously (Perez-Miranda *et al.*, 2007). In summary, CAS medium was prepared by dissolving 0.03025 g CAS in 25 ml of dH_20. Separately, 0.0365 g HDTMA was dissolved into 20 ml of dH_20. With the HDTMA solution stirring, 5 ml of 1 mM $FeCl_3$ (in 10 mM HCl solution) was added, followed by the CAS solution, to achieve a solution which was very dark blue in colour. Simultaneously, 7.5 g Agar No. 1 and 15.12 g Piperazine-N,N'-bis (2-ethanesulphonic acid) or PIPES (free acid), were dissolved in 375 ml dH_20. To facilitate the dissolving of the PIPES powder, 6 ml of 50 % NaOH was added to the solution. The pH of the solution was then adjusted to 6 using 1 M HCl to completely dissolve the reagents. Dye and agar base solutions were sterilised and once cooled to approx. 56 ^oC, the autoclaved dye was carefully poured down the side of the bottle containing the agar solution, and mixed by gentle agitation to avoiding bubble formation.

Isolates of interest were streaked for single colonies onto BHI agar plates (containing antibiotics where required) and incubated at 37 ^oC until growth was evident. Plates were then overlaid with approx. 15 to 20 ml CAS medium. Once set, plates were incubated at 37 ^oC for between 24 and 168 h during which time any colour changes in the blue/green overlay medium were observed.

2.21.2 Siderophore microtitre plate assay

Assay buffer was prepared by stirring 6 ml of 10 mM HDTMA, 0.15 ml 10 mM $FeCl_3$ (in 100 mM HCl), and 7.5 ml CAS into 75 ml piperazine (pH 5.6) solution. The solution was

made up to 100 ml with dH_20 and stored at RT in a plastic, foil-wrapped bottle. The solution was not colloidal and gradually became darker blue as the dye bound to the iron in the solution. A stationary phase culture of *S. uberis* prepared in CDM with 0.5 % hydrolysed casein was used to inoculate, in triplicate, 5 ml of the media of interest (typically CDM with high or low iron concentrations) by diluting 100-fold. After approx. 24 h, when cultures had reached stationary phase of growth, the $OD_{600\ nm}$ values were determined and found to be between 0.6 and 1.0. Cell pellets were produced by centrifugation of cultures at 3,893 × *g* for 30 m. For each replicate of each culture supernatant, 200 µl was deposited into two wells in row A of a 96-well microtitre plate. Tris-buffered saline (100 µl) was aliquoted into all wells of rows B to G of the same plate. Supernatant samples were then serially diluted from rows A to G by pipetting 100 µl from the first row into the next row and so on, with 100 µl being discarded from row G so all wells contained just 100 µl. Into row H, 100 µl of the corresponding un-inoculated medium was added as a control. Finally, to all wells 100 µl of CAS assay buffer was added and the plate, which was then covered with foil, was incubated at RT for 2 h. Quantification was not conducted, as at this stage only the presence or absence of siderophore production was to be determined (by the visualisation of a colour change from blue to pink when siderophore was produced).

2.22 *S. uberis* cell viability assay

AlamarBlue® (Invitrogen) was used to confirm the viability of *S. uberis* cells during growth in various media. Overnight cultures prepared in either BHI broth or CDM were diluted 100-fold into 5 ml of test medium. One hundred µl of inoculated media were transferred into the appropriate wells of a 96-well flat-bottomed microtitre plate along with controls. Plates were incubated at 37 °C for the required period before 10 µl alamarBlue was added to selected wells. Controls comprising medium only, medium with alamarBlue and inoculated medium without alamarBlue were included. Plates were incubated for a further 4 h, after which absorbance at 562 nm was measured, although quantification was not required.

2.23 Protein electrophoresis and western blotting

2.23.1 Preparation of SDS PAGE gel

Mini-Protean3 electrophoresis apparatus (Bio-Rad) was used for protein analysis. Recipes for gels and associated solutions are listed in **Appendix 1**. Glass plates (spacer and short plates) were cleaned, placed together and secured in a cassette which was transferred onto the casting stand. Freshly prepared 15 % (v/v) resolving gel mixture was immediately pipetted between the plates leaving a gap of about 2 cm at the top of the short plate. Isopropanol was pipetted on top of the gel to remove bubbles. Once set, the isopropanol was poured off and the top of the gel rinsed with water. A 5 % (v/v) stacking gel mixture was prepared and pipetted on top of the resolving gel right up to the top of the short glass plate; a 10 well comb was then placed into the gel ensuring no bubbles were introduced. Once the stacking gel had set, the cassette was removed from the casting stand and placed into an electrode unit and then into an electrophoresis tank. The central reservoir was filled with 1× running buffer (2.5 mM Tris, 19.2 mM glycine and 0.01 % (w/v) SDS) whilst the tank was filled up, approx. half way, with the same buffer. The comb was removed from the gel and wells were flushed with running buffer.

2.23.2 Sample preparation, loading and electrophoresis

One volume of 5× denaturing loading buffer was added to 4 volumes of sample. The sample was then boiled at 100 ^{o}C for 3 m, spun briefly, and up to 20 μl of the denatured sample was loaded per well onto the prepared gel. To allow approximation of protein sizes, 20 μl SeeBlue® Plus2 Pre-Stained Standards (Invitrogen) were also loaded. Electrophoresis was then commenced at 100 V/cm until the samples had passed through the stacking gel. The voltage was then increased to 140 V/cm until the bromophenol blue in the loading buffer could be seen leaving the bottom of the gel.

2.23.3 Staining, de-staining and drying

To visualise proteins, the gel was carefully removed from between the glass plates and placed into an appropriate container. The gel was covered with Coomassie Brilliant Blue stain and incubated at RT with gentle agitation overnight. Staining reagent was discarded, and the gel rinsed with, and then covered, with de-stain solution. The gel was then incubated at RT with gentle agitation and the solution changed periodically until background staining was almost clear. At this point, the gel was covered with de-stain solution containing 10 % (v/v) glycerol and the process continued until background

was completely clear. The gel was dried and preserved in cellophane using the DryEase™ mini-gel drying system (Invitrogen).

2.23.4 Transfer to nitrocellulose

For Western blotting, transfer of proteins to nitrocellulose was conducted using the Mini Trans-Blot® Electrophoretic Transfer Cell apparatus (Bio-Rad). Gel cassettes were loaded (black side down) in the following order whilst submerged in transfer buffer (24 mM Tris, 192 mM glycine and 20 % (v/v) methanol); packing mat, filter paper, SDS PAGE gel, 0.45 μm Nitrocellulose sheet (Bio-Rad), filter paper and finally another packing mat. The cassette was squeezed shut and placed into the electrode module which had been filled with transfer buffer. An ice block and stirrer were placed into the module to prevent overheating and a current of 400 mA was applied to the unit for 30 m. After transfer, the membrane was removed, rinsed and placed into blocking buffer (20 mM TBS, pH 7.4, 0.05 % (v/v) Tween-20 and 3 % (w/v) Top Block). The membrane was incubated at RT for 1 h with gentle agitation, and then stored at 4 ^{o}C overnight.

2.23.5 Western Blot

Buffer was poured off membrane and replaced with 15 ml 1 % (v/v) buffer (20 mM TBS, pH 7.4, 0.05 % (v/v) Tween-20 and 1 % (w/v) Top Block). The appropriate primary antibody was diluted 500-fold in the buffer and the membrane incubated at RT with agitation for 1 h. The membrane was then washed three times (each for 5 m) in TBS with 0.05 % (v/v) Tween-20 (TBST). The TBST was discarded and the membrane placed into 15 ml of 1 % (v/v) buffer. The appropriate secondary antibody was diluted 5,000-fold in the buffer and the membrane incubated as previously for a further 1 h. The membrane was then washed with TBST as previously, and placed into 15 ml substrate (1× 3,3-Diaminobenzidine tablet dissolved in dH_20) and further incubated until the positive bands appeared. The reaction was then stopped by washing the membrane briefly in water, then TBST and finally water. The membrane was placed onto filter paper to dry and kept covered to prevent fading of bands prior to photographing.

2.24 Computational and statistical analyses

The genome sequence of *S. uberis* 0140J (Accession number AM946015) was used as a resource for much of the work described in this thesis. The Clone Manager Professional Suite v.9 (Scientific and Educational Software, North Carolina, USA) software package

was used to design oligonucleotide primers, predict PCR products, restriction endonuclease digestion products and ligation products as well as for the alignment of forward and reverse DNA sequences. The Artemis software programme (Rutherford *et al.*, 2000) was used to visualise genome sequencing data generated during this project. BLAST analyses against the NCBI non-redundant database were either conducted through Clone Manager, Artemis or directly from the website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Data was analysed and graphs drawn using Microsoft® Office Excel 2007 (Microsoft® Corporation). Error bars displayed on graphs are presented as the standard deviation of the mean. Contingency tables (two-by-two) were constructed and the significance of the association between the outcomes for two groups determined using the Fisher's exact test, calculated with the VasserStats (©Richard Lowry 1998-2000) online tool (http://faculty.vassar.edu/lowry/fisher.html). Generally, there was considered to be a difference between populations when the probability, or *P*-value, obtained was less than 0.05 (*i.e.* there was 95 % confidence that the identified difference did not arise by chance).

MLST data was submitted to, and certain analyses conducted using the *S. uberis* MLST website (http://pubmlst.org/suberis/), which was developed by Keith Jolley and is sited at the University of Oxford (Jolley *et al.*, 2004). The development of this site is funded by the Wellcome Trust.

Chapter 3: Multi-locus Sequence Typing (MLST) of *S. uberis* mastitis isolates

3.1 Introduction

Sub-typing of disease-causing bacterial populations by genomic characterisation permits observations to be made regarding the evolution and adaptation of pathogens to their environment. Characterisation of the disease causing population during outbreaks, for example, may identify the individual strain responsible for the initial outbreak, and permit observations of its subsequent spread amongst infected individuals (Camargo *et al.*, 2006; Olive and Bean, 1999). Alternatively, genetic typing may identify strains particularly well-adapted to survival within a particular niche, allowing targeting of future vaccines towards these specific strains, or permitting implementation of targeted control programmes (King *et al.*, 2002; van Belkum *et al.*, 2001). Defining the global disease-causing population also allows the efficacy of candidate vaccines to be tested against a panel of diverse strains. Mastitis results in major financial losses for dairy farmers; *S. uberis* is one of the main pathogens associated with bovids (Bradley *et al.*, 2007) and is also, to a lesser extent, associated with mastitis in ewes (Mork *et al.*, 2007). Consequently, characterisation of isolates from mastitis cases would further vaccine development and improve control programmes, increasing animal health and thus profitability.

Failure of biochemical and serological typing to accurately distinguish between strains of *S. uberis* prompted the move to molecular typing. This began in earnest following the reclassification of *S. uberis* type II to *S. parauberis* (Williams and Collins, 1990) and the demonstration that *S. uberis* (formerly type I) was mostly associated with mastitis (Bentley *et al.*, 1993; Jayarao *et al.*, 1991). Simple methods, involving the enzymatic digestion of gDNA, were found, which clearly demonstrated heterogeneity between mastitis derived *S. uberis* isolates (Gillespie *et al.*, 1998; Jayarao *et al.*, 1992; Jayarao *et al.*, 1993; Oliver *et al.*, 1998; Williams and Collins, 1991).

Genomic typing methods, such as random amplified polymorphic DNA (RAPD), repetitive extragenic palindromic typing (REP), ribotyping and PFGE have subsequently been widely utilised to characterise collections of *S. uberis* isolates (Baseggio *et al.*, 1997; Douglas *et al.*, 2000; McDougall *et al.*, 2004; Phuektes *et al.*, 2001; Wieliczko *et al.*, 2002; Zadoks *et al.*, 2005b). Within *S. uberis* mastitis populations many different types were found within herds on individual farms, as well as on different farms in different regions (Baseggio *et al.*, 1997; Douglas *et al.*, 2000; Khan *et al.*, 2003; McDougall *et al.*, 2004; Wieliczko *et al.*, 2002). Furthermore, infection of a single animal with multiple strains has been reported; in one case, in a single animal, four distinct *S. uberis* strains

were found in each udder quarter (Phuektes *et al.*, 2001). Conversely, despite the generally high heterogeneity observed between isolates, some strains were isolated from multiple quarters of the same animal, from different animals on the same farm and even from distinct herds (Khan *et al.*, 2003; McDougall *et al.*, 2004; Phuektes *et al.*, 2001). These observations demonstrate that individual quarters and animals can be infected by exposure to multiple strains from the dairy environment, but the identification of dominant strains implies that cow to cow (or quarter to quarter) transmission is also occurring, although all animals or quarters could be infected by a single strain from the same environmental source. Outbreaks of *S. uberis* mastitis have also been described, and in one case this was attributed to the transmission of a single strain between sub-clinically infected animals via the milking machine (Zadoks *et al.*, 2003). These results suggest a complex model of disease transmission by *S. uberis*, although the dynamics of transmission remain to be characterised in detail.

The typing studies discussed thus far rely upon PCR amplification and/or DNA digestion, and as such are affected by experimental conditions and equipment. The techniques vary, and rely heavily upon manual assignment of banding patterns, even when using computer programmes. Consequently, the results obtained are somewhat user-dependent and open to interpretation; variability between laboratories is therefore likely, even when highly reproducible results are achieved by an individual user. The comparability of data between researchers is thus limited (Maiden *et al.*, 1998; van Belkum *et al.*, 2007), and analysis of *S. uberis* mastitis isolates using RAPD typing clearly demonstrated this point; a characteristic band described in a previous study was not found in a subsequent study (Gillespie *et al.*, 1998; Wieliczko *et al.*, 2002). Most of these techniques presume isolates with minimal banding pattern differences to be closely related (Tenover *et al.*, 1995), however, as decisions on banding patterns are open to interpretation, the presumed links between isolates may also be tenuous. To unambiguously further explore the population genetics of *S. uberis* strains from cases of mastitis; researchers have begun utilising an alternative technique called multi-locus sequence typing (MLST).

The MLST procedure was developed by Maiden *et al.* in 1998, being essentially an updated version of the multi-locus enzyme electrophoresis method, where determination of the electrophoretic mobilities of several housekeeping genes has been replaced by the sequencing of approx. 500 bp regions within these genes (Maiden *et al.*, 1998; Selander *et al.*, 1986). Unique sequences at each locus are assigned novel allele numbers, thus when all genes have been sequenced, allelic profiles are obtained.

Sequence types are then assigned to each unique allelic profile and these can be compared to determine relatedness of different isolates. To demonstrate the technique, *Neisseria meningitidis* isolates from healthy carriers or patients with invasive disease were characterised, and MLST distinguished between isolates from the major subgroups associated with epidemic meningitis (Maiden *et al.*, 1998). MLST has subsequently been utilised to analyse the genomic diversity amongst isolates from a wide range of bacterial species, including the pig pathogen *S. suis*, the endosymbiont *Wolbachia pipientis* and non-pathogenic *Medicago*-nodulating rhizobia (Baldo *et al.*, 2006; King *et al.*, 2002; van Berkum *et al.*, 2006).

The main advantage of the MLST protocol is that sequencing of internal gene regions determines the exact nucleotide differences between alleles, permitting unambiguous observations of relatedness to be made. Data can also be stored electronically and thus readily compared between laboratories. The internet has been effectively utilised to develop MLST databases, such as mlst.net/mlstdbNet and PubMLST (Aanensen and Spratt, 2005; Jolley *et al.*, 2004) which are ideal for sharing MLST data. New sequences can then be compared to a database, with novel alleles and STs being assigned by the database curator, at their discretion, providing consistency between datasets and allowing standardised results from around the world to be compared. PubMLST databases exist for many bacterial species, including *S. aureus*, *S. agalactiae* and *S. uberis* (Coffey *et al.*, 2006). Furthermore, nucleotide sequences and allelic profiles obtained from MLST experiments can be readily loaded into computer programmes such as BURST (based upon related STs) or eBURST (an updated version), to define biologically meaningful clusters of STs known as clonal complexes (CC), permitting hypotheses regarding evolutionary descent to be made (Feil and Enright, 2004; Feil *et al.*, 2004; Hall and Barlow, 2006; Spratt *et al.*, 2004). The START (Sequence Type Analysis and Recombinational Tests) programme is also available, which comprises numerous functions allowing the user to explore allele diversity, polymorphisms and recombination within a data set (Jolley *et al.*, 2001).

The selection of MLST gene targets, as with any typing scheme, is probably the most important stage in the MLST process and as such is much disputed (Coffey *et al.*, 2006; van Belkum *et al.*, 2007; Zadoks *et al.*, 2005a). Housekeeping genes involved in routine cell functions are generally highly conserved, accumulating genetic variation slowly, providing an opportunity to explore global epidemiology over a long period of time. Conversely, genes encoding virulence factors and cell-surface proteins are subjected to high selective pressures, forcing them to emerge and evolve quickly, offering an

opportunity to closely explore local epidemiology over a short period, such as during a disease outbreak. Therefore, the selection of genes should be based upon the question to be addressed regarding the specific bacterial population being analysed. The ability of MLST to discriminate between isolates of *Salmonella enterica* was enhanced to allow greater discrimination than any previous MLST or PFGE scheme, by using a combination of housekeeping and virulence genes, termed MLST-v for MLST virulence (Tankouo-Sandjong *et al.*, 2007). Similarly a Multi-Virulence-Locus Sequence Typing (MVLST) scheme for *Listeria monocytogenes* offered improved discrimination over MLST or PFGE (Zhang *et al.*, 2004). Generally, however, PFGE remains more discriminatory than MLST (Grundmann *et al.*, 2002; Rabello *et al.*, 2007; Smith *et al.*, 2005), as this technique analyses the entire genome and can thus highlight micro-variation within rapidly evolving genes as well as smaller variations between neutral housekeeping genes (Rato *et al.*, 2008; van Belkum *et al.*, 2007). The success of a typing technique varies widely however, depending upon the species or the sub-population analysed and the restriction enzymes, primers or target genes used. For example, MLST was shown to be more discriminatory than PFGE for typing *S. aureus* from dairy sheep (Vautor *et al.*, 2005), but in a different study *S. uberis* isolates sharing the same ST exhibited different PFGE types (Rato *et al.*, 2008).

The first MLST scheme designed to explore the epidemiology of *S. uberis* combined virulence and housekeeping genes, and included genes whose products are of interest as potential sub-unit vaccine candidates (Zadoks *et al.*, 2005a). The MLST scheme was more discriminatory than ribotyping and results more closely corresponded to epidemiological origin, with 40 STs identified from 50 US and Dutch isolates (Zadoks *et al.*, 2005a). Subsequently, another MLST scheme was developed in 2006 based on housekeeping genes only, which discriminated 160 *S. uberis* mastitis isolates from 6 UK herds into 57 STs (Coffey *et al.*, 2006). Most STs (n=39) represented just a single isolate, but the most common, ST 5, comprised 21 isolates and was identified as the founder of a major UK lineage, or CC, incorporating 70 % of the population examined (Coffey *et al.*, 2006). Subsequently, just 4 STs were identified, following a later sampling of mastitis cases from the same herd, which had also been identified during the first sampling period, but interestingly 3 of these STs were from the dominant CC of the first analysis (Pullinger *et al.*, 2007). The housekeeping MLST scheme was used to set up an online *S. uberis* MLST database (pubmlst.org/suberis/) which has subsequently been utilised by researchers around the world (Lopez-Benavides *et al.*, 2007; Pullinger *et al.*, 2007; Pullinger *et al.*, 2006; Rato *et al.*, 2008; Tomita *et al.*, 2008). Recently, 21 selected diverse strains were typed using both described *S. uberis*

MLST schemes, and the protocol of Coffey *et al.* (2006) discriminated between two isolates where the scheme of Zadoks *et al.* (2005a) did not (Lang *et al.*, 2009). Approximate location of MLST gene targets from both schemes within the *S. uberis* genome are shown in **Figure 3.1**.

The value of a shared database was subsequently demonstrated, as two distinct CCs were identified by MLST amongst *S. uberis* isolates from New Zealand, but none of the STs found were the same as those from the UK, although a few STs belonged to the large UK CC identified (Pullinger *et al.*, 2006). Two STs from an Australian collection were, however, found to be identical to STs identified in either the UK or New Zealand (Tomita *et al.*, 2008). The *S. uberis* populations in different countries thus generally appear to be divergent, as was demonstrated previously by DNA fingerprinting studies of isolates from the US and New Zealand (Gillespie *et al.*, 1998). Two clusters of STs from six Australian farms were also identified; one was highly associated with isolates from clinical and sub-clinical mastitis whilst the other was significantly associated with low SCC, suggesting that some strains may exhibit increased pathogenicity (Tomita *et al.*, 2008). A further study, failed, however, to demonstrate niche adaptation, as no ST or CC was enriched in just the environment, the milk or on the cow (Pullinger *et al.*, 2006). This observation was in agreement with a previous ribotyping study of environmental, faecal and milk isolates (Zadoks *et al.*, 2005b). Several STs were isolated however, more than once from farm tracks as well as from intra-mammary infections, suggesting a link between environmental exposure and infection of the mammary gland (Lopez-Benavides *et al.*, 2007); although, it is equally plausible that the farm tracks were contaminated by mastitic milk from infected udders.

As early as 1989 it was demonstrated that over an extended period (four weeks) a single cow quarter could be infected with the same *S. uberis* strain (Hill and Leigh, 1989). Later, PFGE showed that 55 % of quarters were infected with the same type at 28 day intervals (McDougall *et al.*, 2004). During a six week period within a single lactation, 41 of 47 pairs of isolates were similarly observed to be of the same PFGE type, whilst of 13 quarters which remained infected over two lactations, different strains (or subtypes) were found in all cases, suggesting re-infection with different strains between lactations (Phuektes *et al.*, 2001). This was in contrast to an earlier demonstration that the same subtype (as determined by DNA fingerprinting) persisted in two quarters over two lactations (Oliver *et al.*, 1998). Most recently, MLST identified that approx. 70 % of long duration *S. uberis* infections were due to continual infection by the same ST (Pullinger *et al.*, 2007). The observations were based on typing pairs of

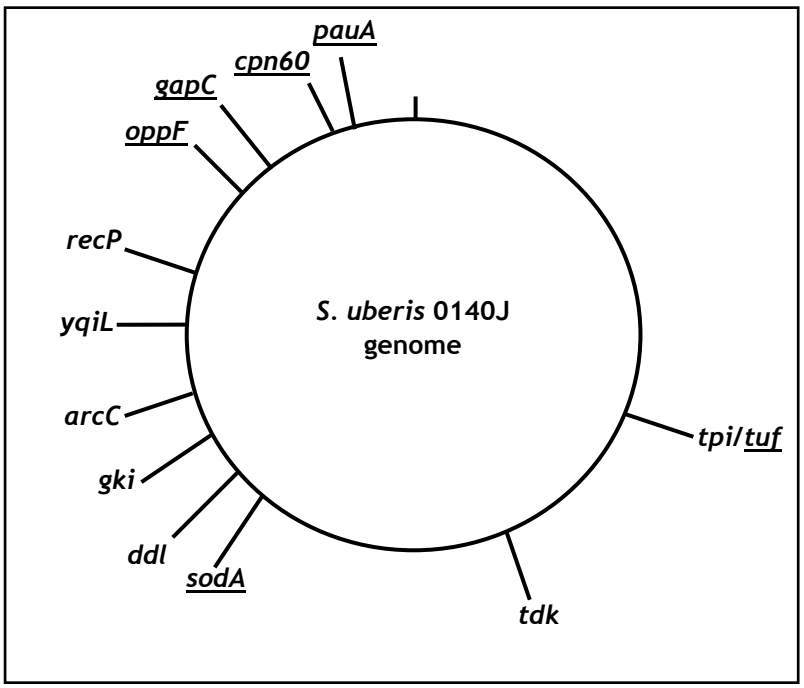


Figure 3.1: Location within the *S. uberis* genome of MLST targets. Approximate locations, within the *S. uberis* 0140J genome, of the genes which have been utilised as targets for MLST schemes. Targets used in the protocol of Zadoks *et al*. (2005a) are underlined whilst those used by Coffey *et al*. (2006) are not. Figure adapted from Lang *et al*., 2009.

isolates which were, in some cases, over 200 days apart; thus, typing of additional samples at intervals between these two points would further confirm that the single strain persisted during the entire period and that re-infection with the same strain did not occur between samplings. Such an observation has been made previously using RAPD fingerprinting of quarter isolates at 3 weekly periods over 18 months (Zadoks *et al.*, 2003). Evidence thus suggests that persistent *S. uberis* infections are common; but again, no specific ST could be linked to either persistent or brief *S. uberis* infections (Pullinger *et al.*, 2007). Host factors are thus likely to impact upon infection duration, particularly as in this study no antibiotics were given (Pullinger *et al.*, 2007). As such, in this case, extended infections were not due to an increased ability of bacteria to resist antibiotics *in vivo*, instead these strains may have adapted to resist host defences.

The large numbers of STs identified in these MLST studies supports the theory that *S. uberis* is transmitted to cows predominantly from the environment; however, MLST results from a Portuguese study were quite different. No STs were found on more than one farm and all six *S. uberis* isolates from one farm shared the same ST; whilst on another farm, half of the isolates (n=9) shared one additional ST (Rato *et al.*, 2008). The authors observed that in Portugal contagious pathogens are still dominant, with *S. aureus* and *S. agalactiae* accounting for much higher percentages of mastitis cases than in the UK (Rato *et al.*, 2008). It is thus feasible that *S. uberis* is similarly being spread from cow to cow due to lower hygiene standards, producing typing results similar to those seen with *S. agalactiae* mastitis isolates (Baseggio *et al.*, 1997).

No typing information could be found regarding *S. uberis* isolates from different hosts but several typing studies of this kind have been conducted using *S. aureus* mastitis isolates. Some PFGE pulsotypes were unique to *S. aureus* mastitis isolates from sheep, cows and goats whilst the most dominant pulsotypes, or the major clone, were found in all three host species (Aires-de-Sousa *et al.*, 2007; Mork *et al.*, 2005). Two closely related, dominant STs from sheep, goats and human isolates were also identified by MLST from French farms (Vautor *et al.*, 2005). Whole genome scanning and comparative genome hybridizations have found evidence of host adaption amongst *S. aureus* genomes, although, surprisingly, heterogeneity was highest within the core genome of host-specific isolates (Ben Zakour *et al.*, 2008).

Genomic characterisation of *S. uberis* mastitis isolates using PCR, PFGE and MLST has shown that the epidemiology of *S. uberis* is complex, with substantial heterogeneity

evident within and between loci (Coffey *et al.*, 2006; Zadoks *et al.*, 2005a). In most cases, a diverse population of strains was shown to be associated with bovine mastitis. While no individual strain was identified with clearly increased pathogenic potential, which could be targeted through specific mastitis control programmes, a collection of dominant strains in the UK, Australia and New Zealand have been characterised (Pullinger *et al.*, 2007; Pullinger *et al.*, 2006; Tomita *et al.*, 2008). Evidence has been given for certain groups of strains having increased pathogenic potential, whilst both cow to cow and environmental transmissions of *S. uberis* have been implied.

The aim of this study was to utilise MLST for the characterisation of *S. uberis* isolates, to explore the heterogeneity between and within different sub-populations and identify if any particular strains were adapted to specific niches. A panel of *S. uberis* isolates were received from the UK and Italy, representing two distinct sub-collections; UK isolates were characterised either as persistent or non-persistent depending on the response to antibiotic therapy (although all isolates were equally sensitive to antibiotics *in vitro*), while Italian isolates were derived from either bovine or ovine mastitis cases. The identification of strains associated with persistence, or differences between strains from different host species were thus of particular interest. To the author's knowledge, this is the first such comparison of isolates from different species. Data was latterly to be submitted to the MLST database and compared to the global *S. uberis* population. Additional aims were to explore the frequency and diversity of the *pauA* and *gapC* genes within mastitis populations, and determine if sequence data could offer any contribution to MLST analysis. Finally, MLST results were to be utilised for the selection of a sub-panel of genetically diverse *S. uberis* strains for proteomic analysis to be completed within the mastitis group, as well as for phenotypic analysis conducted in later chapters of this work.

3.2 Results

3.2.1 *S. uberis* panel and initial species confirmation

A total of 246 *S. uberis* mastitis isolates were received from the UK (n=196) and Italy (n=50) and comprised samples from bovine and ovine mastitis cases, as well as samples from apparently persistent or non-persistent infections. Two UK and one Italian isolate failed to grow satisfactorily following resuscitation in BHI broth, so these samples could not be included in the study. Colony morphology was observed on BA plates, with small, dry, well-defined, non β-haemolytic, white colonies considered to be the normal appearance for *S. uberis* (**Figure 3.2 A**). Isolates lacking this characteristic appearance were further scrutinised using the API 20 Strep test kit. Four additional isolates were thus excluded for failing to be identified as *S. uberis* or for exhibiting gross contamination. Further isolates were excluded at later stages in the MLST process (discussed later).

A quarter of the collection of isolates displayed larger, less-defined, mucoid, grey/white colonies (**Figure 3.2 B**) but these isolates were confirmed to be *S. uberis* following scrutinisation by API testing. The observed mucoid appearance is likely to represent high levels of capsule production by these isolates under these particular growth conditions.

3.2.2 Genomic DNA extraction and PCR

Initially, four arbitrarily-selected *S. uberis* mastitis isolates, in addition to the reference strains 0140J and 20569 and an *Enterococcus durans* isolate (used as a negative control), were utilised to optimise DNA extraction and PCR protocols. High quality gDNA was then extracted from the remaining *S. uberis* mastitis isolates using high-throughput methods. The success of the extractions and the resulting quality of the nucleic acid preparations was determined by electrophoresis of 10 DNA samples picked arbitrarily from each 96-well plate. In all cases a single clear band greater than 10 kb (the largest weight marker in the DNA ladder) with no smearing was produced, indicating no DNA degradation or RNA contamination (data not shown). Spectrometric analysis was conducted for all samples, from each plate, and gDNA was diluted (to a final concentration of 100 ng/μl) for use as the template for amplification of MLST targets. The standardised protocol available on the *S. uberis* MLST database (http://pubmlst.org/suberis/) was utilised to allow comparison of results to the currently available data. Annealing temperatures for the amplification of *arcC*, *ddl*,

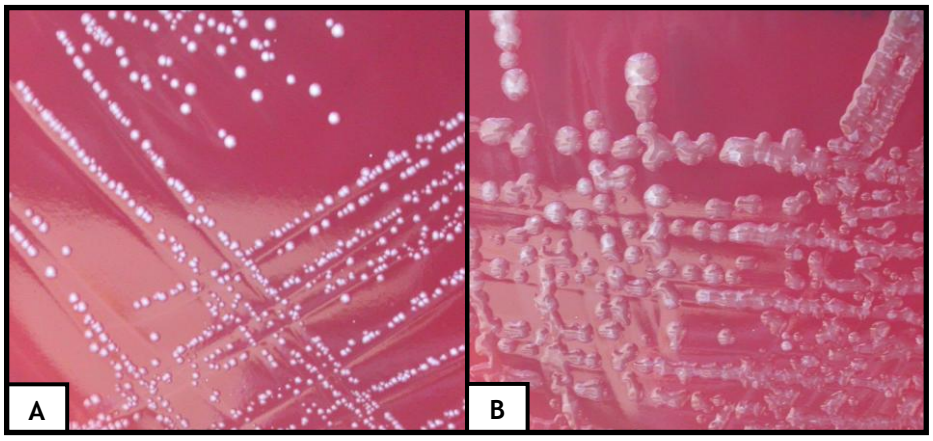


Figure 3.2: Characteristic *S. uberis* colony morphology as seen on blood agar plates. The typical dry *S. uberis* colony morphology observed (A) is compared to the mucoid morphology also frequently visualised, representing highly capsular *S. uberis* isolates (B).

gki, recP, tdk, tpi and *yqiL* gene regions were optimised using gDNA from the seven 'test' isolates described above. Amplification of all seven genes was successfully achieved following PCR using gDNA from the six *S. uberis* isolates as templates. Alternatively, 5 gene regions could not be amplified from gDNA of *E. durans* (**Figure 3.3**).

Whilst a strong band of the predicted size (793 bp) was obtained following amplification of the *tdk* gene region, there was also a noticeable second band seen at approx. 400 bp (**Figure 3.3**). Despite further optimisation of the PCR conditions and additional purification of the PCR products, this band could not be satisfactorily removed. By referring to the *S. uberis* 0140J genome sequence (Accession No. AM946015), novel primers were designed to amplify an equivalent region of the *tdk* gene, ensuring that the sequenced PCR product would still incorporate the 500 bp region utilised by the online MLST scheme. These primers (*tdk*TL F and *tdk*TL R) amplified a single product of the predicted size (761 bp) with no additional band present (data not shown). These primers were thus used for amplification of the *tdk* region from gDNA of remaining *S. uberis* isolates. Amplification of the seven PubMLST target regions was completed for all UK and Italian *S. uberis* isolates. Twenty-one isolates failed to amplify at least two, but in most cases all, of the target gene regions. API tests confirmed these isolates were not *S. uberis* and they were excluded from the study. Most excluded isolates were identified as *Enterococcus* (*faecium, faecalis* and *durans*).

To confirm putative species identification of the remaining isolates, PCR amplification of the species specific region of the *S. uberis* 16S rRNA gene (Hassan *et al.*, 2001) was conducted. The protocol was first optimised using *S. uberis* reference strains 0140J and 20569 where a single band of approx. 445 bp was amplified using primers 16 S. *uberis* F and 16 *S. uberis* R. Purified PCR products were sequenced and a BLAST analysis search against the NCBI non-redundant database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) confirmed that 422 bp of this sequence from both strains displayed 100 % nucleotide identity to the 16S ribosomal RNA sequence of *S. uberis* strain 0140J (AM946015). Successful amplification of a 445 bp product was thus subsequently used to confirm species identity of all remaining *S. uberis* isolates from which seven MLST target genes had been successfully amplified (data not shown). Two isolates, T2-65 and T2-77 were exceptional in that they produced only faint bands following initial and repeat PCR; as this result was ambiguous, these isolates were excluded from subsequent MLST analyses. In total, 167 UK and 49 Italian isolates were analysed, and, in most cases, PCR products from all seven gene targets were sequenced; the exceptions in which PCR was

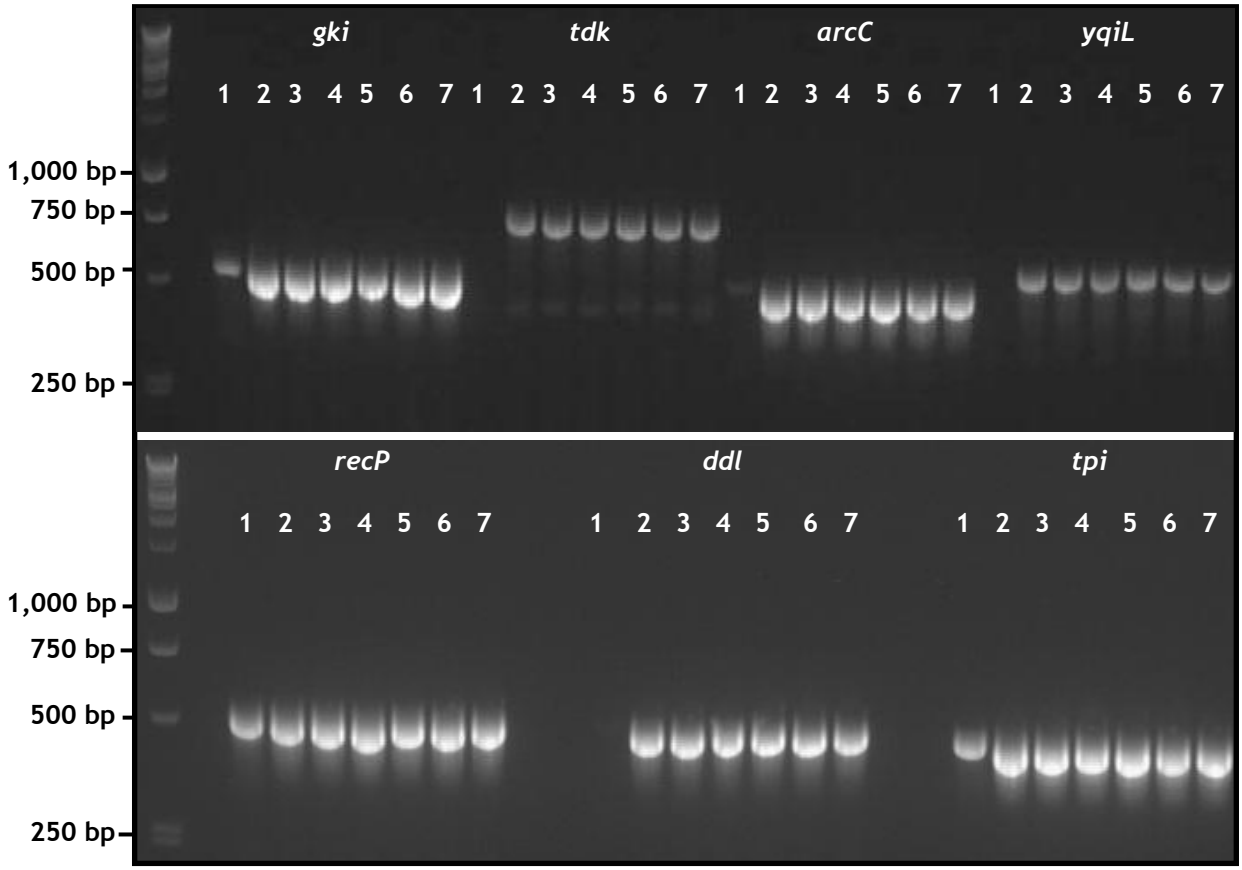


Figure 3.3: Amplification of seven MLST gene targets from gDNA of six *S. uberis* and one *E. durans* isolate. PCR amplified MLST gene targets *arcC*, *ddl*, *gki*, *recP*, *tdk*, *tpi* and *yqiL* were analysed from test panel; T1-2 *E. durans* (1), T1-3 (2), T2-1 (3), T3-1 (4), T3-32 (5), 0140J (6) and20569 (7). A faint additional band of approx. 400 bp can be seen following amplification of the *tdk* gene region.

not successful are discussed below.

It was not possible to amplify the *yqiL* gene from four *S. uberis* isolates, although the remaining MLST targets were successfully amplified from these isolates; these were T1-59, a bovine UK isolate, and I14, I34 and I49, three Italian isolates, one from ovine and two from bovine mastitis cases. API testing and 16S rRNA PCR confirmed the species identity of these isolates, however, despite optimisation of the amplification conditions, no *yqiL* product could be obtained (**Figure 3.4**). Upon consultation with the literature, it was found that *yqiL* deficient *S. uberis* isolates have been identified previously and that a gene of similar function is located elsewhere in the *S. uberis* genome (Tomita *et al.*, 2008). The alternative gene (*thlA*) found at bps 1795067-1796317 on the *S. uberis* genome has now been annotated and the gene product designated acetyl-coA acetyltransferase, whilst the *yqiL* gene is located at bps 1391827-1393014 and its product is designated acetyl-coA acetyltransferase 2. It was found that a 641 bp internal region of *thlA* was successfully amplified by PCR from gDNA of both *yqiL* positive and all *yqiL* negative isolates identified in this study, using primers Acetyl F and Acetyl R, suggesting that gene conservation in this region may be higher than at the *yqiL* region.

The sequence diversity at the acetyl-coA acetyltransferase region was briefly investigated by sequencing the PCR products from the seven isolates tested. Sequences are listed in **Appendix 2**. Within 527 bps of sequence, 8 variable nucleotides were identified from 7 isolates.

Production of PauA or GapC proteins is hypothesised to play a role in the virulence of *S. uberis*, and thus these proteins have been assessed as sub-unit vaccine candidates. The *gapC* gene was successfully amplified from gDNA of all *S. uberis* isolates by PCR, whilst several isolates, failed to amplify *pauA* as discussed in more detail in **Section 3.2.3**. Due to cost restraints, only *pauA* and *gapC* PCR products from the 49 Italian isolates were sequenced.

3.2.3 Plasminogen activator A (*pauA)* observations

Electrophoresis of *pauA* PCR products identified eight *S. uberis* isolates which produced either no band, or a very faint band, such that sequencing of these products was not possible. PCR was repeated using less stringent conditions but a product could still not be amplified from the gDNA of these isolates (**Figure 3.5**). It was of significant interest

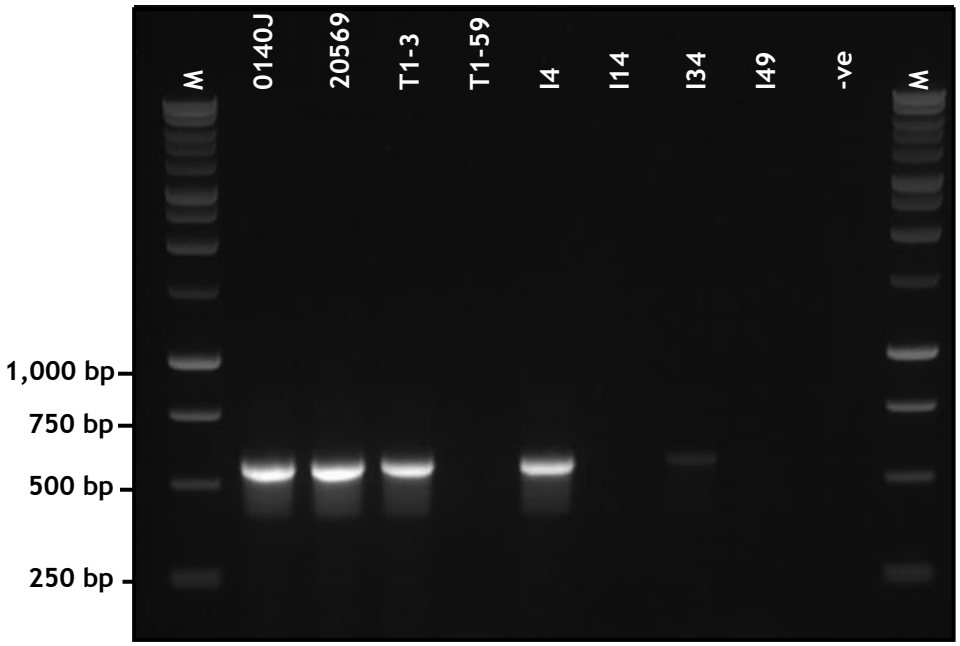


Figure 3.4: *S. uberis* isolates from which the *yqiL* gene could not be amplified. Despite optimisation and significant relaxation of PCR conditions, the *yqiL* 'housekeeping gene' could not be amplified satisfactorily from gDNA of UK isolate T1-59 and Italian isolates I14, I34 and I49.

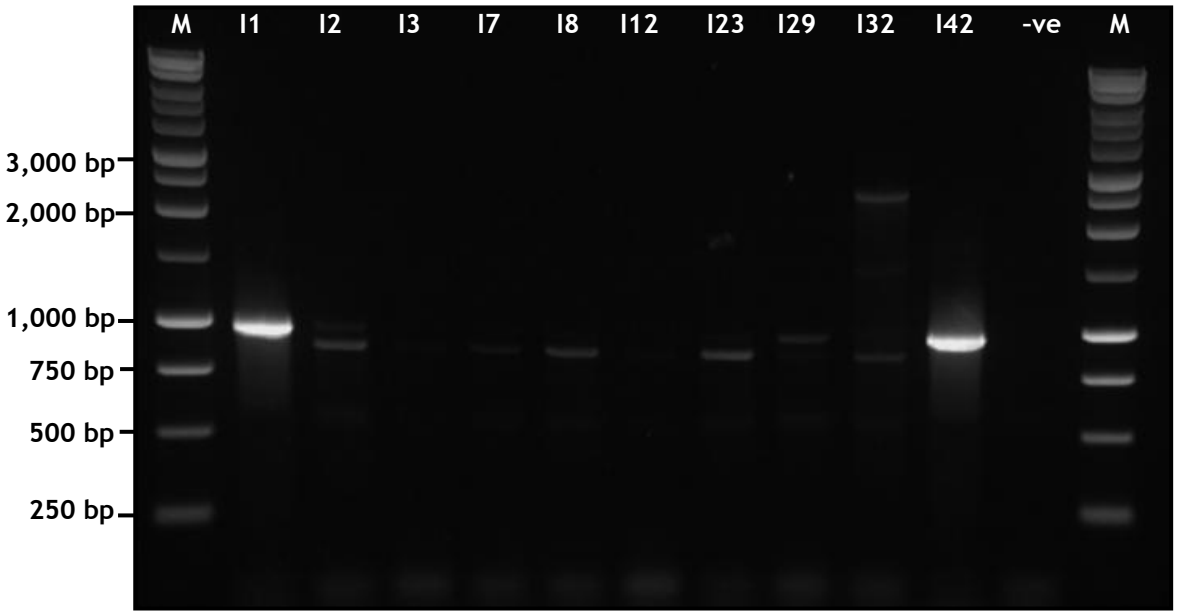


Figure 3.5: *S. uberis* isolates from which the *pauA* gene could not be amplified by PCR. A *pauA* product of the predicted size (976 bp) was amplified from *S. uberis* isolates I1 and I42 (positive controls) and sequenced successfully. In contrast, the *pauA* region could not be amplified from isolates I2, I3, I7, I8, I12, I23, I29 and I32 using the same primers (*pauA* F & *pauA* R) and relaxed amplification conditions.

that all isolates were from the Italian collection (I2, I3, I7, I8, I12, I23, I29 and I32), and additionally, that 5 of the 8 isolates were from ovine mastitis cases. All isolates from which *pauA* could not be amplified, did not, however, originate from a single specific region.

In a Danish *S. uberis* isolate from which *pauA* could not be amplified, this gene was shown to be directly replaced by another gene encoding a broader spectrum plasminogen activator, termed *pauB* (Ward and Leigh, 2002). Primers flanking this region (ER45 & ER46) were used to amplify a PCR product of approx. 2.1 kb from all 8 *pauA*-negative isolates from this study, whilst a product of approx. 1.2 kb was amplified from control isolates in which *pauA* had previously been sequenced (data not shown). Sequencing of the 2.1 kb PCR fragments, followed by BLAST analysis confirmed that sequences obtained from this region in all 8 isolates matched *pauB,* with high homology to the *pauB* sequence in the GenBank database (Acc. No. AJ314852). The identity of the 1.2 kb product from control isolates was similarly confirmed as *pauA*.

Primers (*pauB* F & *pauB* R) were designed, based on obtained *pauB* sequences, to amplify a 1,261 bp internal region of the *pauB* gene from all 8 isolates. Sequencing of PCR products demonstrated more than 95 % nucleotide sequence conservation, and similarly, the translated products of the sequenced fragments shared an equivalent level of homology with PauB (Acc. No. CAC85651). In the eight Italian isolates, *pauA* was thus confirmed to have been directly replaced by *pauB*. The frequency of *pauB* was higher in ovine isolates (5 of 14 or 36 %) than in bovine isolates (3 of 35 or 9 %) and using the Fisher's exact test, this was found to be statistically significant (P=0.033). Four different *pauB* alleles were identified within the 1,087 bp region sequenced (**Appendix 3**). Interestingly, allele 1 was shared by ovine isolates I2, I3, I8 and I12 with the remaining ovine sequence from isolate I7 also being identical except for a 15 bp repeat region between base pairs 775 and 776 (the I7 sequence was thus termed allele 2). Allele 3 was found in bovine isolates I23 and I32 and differed from allele 1 at 11 nucleotides. Isolate I29 displayed allele 4, this sequence diverging from allele 3 at 5 nucleotides and from allele 1 at 10 nucleotides. Bovine isolates displayed the greatest level of homology to the published *pauB* sequence (99 % at the amino acid level), with allele 4 comprising just 3 nucleotide changes and allele 3 having 6 changes, whilst ovine allele 1 displayed 11 nucleotide differences. All Italian alleles did however share two nucleotide changes at bps 1097 and 1113 from the published *pauB* sequence. A phylogenetic tree was created to demonstrate the homology between *pauB* sequences (**Figure 3.6**)

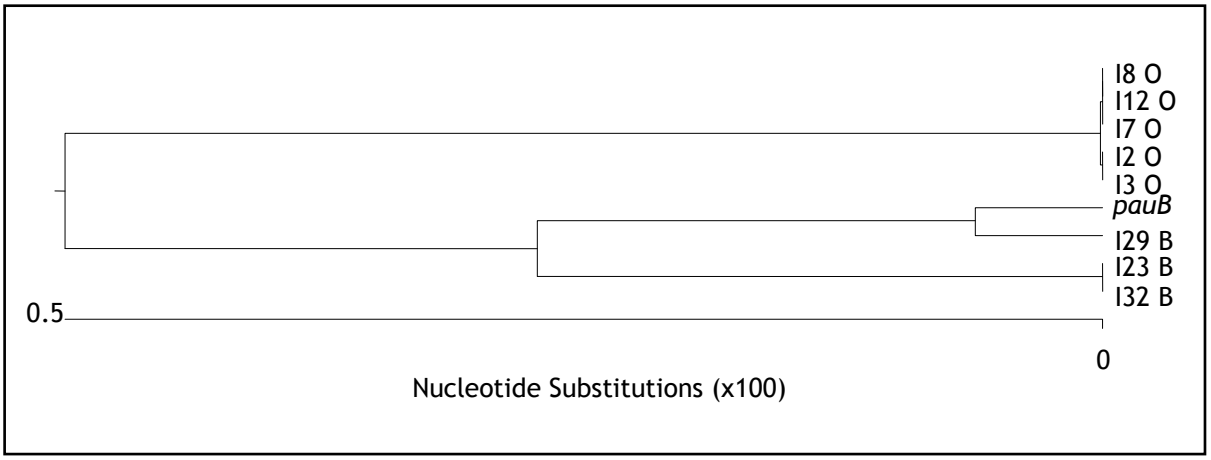


Figure 3.6: Phylogenetic tree of aligned *pauB* sequences from Italian *S. uberis* mastitis isolates. The phylogenetic tree was generated by aligning nucleotide sequences from the *pauB* locus obtained from *S. uberis* mastitis isolates against the same region of the *pauB* gene (Acc. No. AJ314852) from a bovine *S. uberis* isolate that originated from Denmark (SK880). This was done using the ClustalW method in the Lasergene MegAlign Software (DNASTAR, Inc.). The tree illustrates that sequences from all ovine (O) isolates are homologous, whilst sequences from bovine (B) isolates more closely matched each other and the sequence from the Danish isolate.

3.2.4 Alignment of PCR sequences and allele assignment

Forward and reverse sequences for each MLST gene from individual isolates were aligned using Clone Manager, where differences were observed, these regions on the trace files were scrutinised and where necessary sequencing was repeated. A single sequence was thus produced, incorporating both forward and reverse sequences.

DNA sequences were exported from Clone Manager in FASTA format using the programme's multiple file transfer function. The gene sequences from all isolates were imported into the SeqMan programme and compared with allele 1 (for that locus) from the *S. uberis* MLST database. The "assemble" command was used to align sequences and the ends were trimmed to the same size as allele 1. The alignment was saved as a FASTA file then converted to a text file. This was copied directly into the single locus batch query tool in the profiles database section accessed via the *S. uberis* PubMLST website, selecting the appropriate gene for comparison. An allele number for the gene region was then assigned for each isolate by comparison to existing database alleles, or where alleles were novel, trace files were submitted to, and new allele numbers assigned, by the database curator. This process was completed for each gene region allowing determination of seven digit allelic profiles for each isolate. Allele sequences and profiles for individual study isolates can be accessed by searching or browsing the isolates database (http://pubmlst.org/suberis/) for ID numbers 619 to 836. Information regarding allele heterogeneity is provided in **Table 3.1**. The total number of alleles identified at each locus varied from four at the *recP* locus to thirty at the *tdk* locus. It was interesting to note that the same 4 *recP* alleles were found in both the UK and Italian collection, this was not the case with any of the other alleles. It seems that *recP* is highly conserved amongst *S. uberis* isolates and a total of just 17 *recP* alleles are listed in the MLST database. Going to the opposite extreme, 17 *tdk* alleles were identified in the UK collection and 19 in the Italian collection; as with most of the loci, several alleles were found in both collections, whilst a few alleles were unique to each set. The *tdk* locus is clearly highly variable in *S. uberis* with a total of 63 alleles identified on the database. Interestingly, at the *gki* locus over half the UK collection exhibited allele 2, whilst this was not found in any of the Italian isolates. The mean number of alleles at each locus was 16 and the number of novel alleles identified at each locus was higher in the Italian collection.

Table 3.1: Heterogeneity observed at the *S. uberis* MLST gene loci.

Locus	No. of alleles (novel alleles)	Polymorphic sites (total mutations) [A]	Synonymous changes [A]	Non-synonymous changes [A]	Recombination events [A]	dN/dS ratio [B]
UK isolates						
arcC	11 (0)	8 (8)	7	1	2	0.043
ddl	13 (4)	13 (13)	8	4	1	0.154
gki	11 (2)	9 (9)	9	0	1	>0
recP	4 (0)	4 (4)	3	1	0	0.104
tdk	17 (4)	22 (23)	21	2	4	0.026
tpi	7 (2)	6 (6)	2	4	0	0.647
yqiL	8 (0)	8 (8)	6	2	1	0.096
Italian isolates						
arcC	13 (4)	11 (12)	11	1	1	0.028
ddl	11 (4)	13 (13)	10	2	2	0.062
gki	10 (6)	10 (10)	10	0	1	>0
recP	4 (0)	4 (4)	3	1	0	0.104
tdk	19 (11)	26 (27)	25	2	6	0.022
tpi	6 (3)	4 (4)	2	2	0	0.324
yqiL	11 (6)	11 (12)	8	4	2	0.146
gapC	16 (8)	16 (16)	12	4	0	0.105
pauA	7 (2)	14 (14)	3	8	2	0.757
All isolates						
arcC	16 (4)	12 (13)	12	1	1	0.025
ddl	18 (8)	17 (17)	11	5	2	0.140
gki	18 (8)	16 (16)	16	0	2	>0
recP	4 (0)	4 (4)	3	1	0	0.104
tdk	30 (15)	28 (29)	27	2	6	0.020
tpi	9 (5)	8 (8)	3	5	0	0.540
yqiL	16 (6)	14 (15)	9	6	3	0.195

[A] Values were calculated using DnaSP default parameters. Synonymous and non-synonymous changes were identified after assigning coding regions for each gene. The minimum number of recombination events was determined in DnaSP by the method of Hudson and Kaplan (1985). Values were confirmed by equivalent START2 analysis.

[B] dN/dS values were determined using DnaSP by dividing dN by dS; where dN is the average number of non-synonymous substitutions divided by the number of non-synonymous sites within the collection and dS is the equivalent value with synonymous substitutions and sites.

The number of polymorphisms and synonymous and non-synonymous changes were identified at each allele using DnaSP and START2 (Table 3.1). At the *arcC* and *tdk* loci, on at least one occasion, three different nucleotides were identified at a single variable site. In all other cases only two nucleotides were found at variable sites. As expected, most nucleotide substitutions resulted in synonymous changes which did not affect the translated sequence. The following interesting points were, however, observed: Despite *tdk* possessing the highest number of alleles and polymorphic sites (n=28), only 2 non-synonymous mutations were seen within this locus. Conversely, at the *tpi* locus, of just 8 polymorphisms, 5 resulted in non-synonymous changes. At the *yqiL* locus a high number of non-synonymous mutations were also identified. The *gki* locus was the only region displaying no non-synonymous changes, despite a total of 16 polymorphic sites.

The ratio of non-synonymous to synonymous substitutions is utilised as a basic measure of the strength of selection pressure, with positive selection evident where the dN/dS value is greater than 1. Values for dN/dS are given as >0 when there are no non-synonymous changes; in these cases a single amino acid change is clearly detrimental to the function of the protein, hence isolates exhibiting these changes are not identified as resulting cells are not viable, this was only observed at the *gki* locus. For the MLST targets the values were mostly in the region of, or below, 0.1; which would be expected for 'housekeeping genes' which are not supposed to be subjected to high levels of selection pressure. Unexpectedly, the highest dN/dS value identified was for the putative virulence gene, *pauA* (dN/dS=0.757), indicating that this gene is subjected to strong selection pressure. Interestingly, the dN/dS value for *tpi* was also very high, especially amongst the UK isolates, suggesting that this gene is also experiencing high levels of selection pressure, uncharacteristic of a housekeeping gene. Furthermore, the dN/dS value calculated for the *yqiL* gene from the UK and Italian collections was also fairly high (0.195). The START and DnaSP programmes were also used to predict the minimum number of recombination events which have occurred at each locus, with the highest value being identified within the *tdk* locus (n=6), whilst no recombination events were predicted to have occurred at the *recP*, *tpi* or *gapC* loci.

3.2.5 Sequence type assignment

Sequence types for each isolate were identified from allelic profiles of seven housekeeping genes. Profiles were compared to the MLST database and either assigned to existing STs, or where allelic profiles were novel, new STs were assigned by the database curator. In this study, an allelic profile of 1, 1, 1, 1, 1, 1, 1 was obtained for

the positive-control, reference strain, 0140J, which was equivalent to ST 1 which had been determined for 0140J during a previous study (Coffey *et al.*, 2006). Sequence types for all UK and Italian isolates are shown in **Table 3.2**.

In the entire collection of 216 isolates, 99 distinct STs were identified. Within the UK collection of 167 isolates a total of 68 STs were represented, whilst within the Italian collection of 49 isolates 31 STs were identified. Four isolates were not assigned STs as they failed to amplify the *yqiL* gene, as discussed in **Section 3.2.2**. No single ST was found simultaneously within both the UK and Italian collections. The most common ST found in the UK was ST 5 (n=17), whilst in Italy ST 305 predominated (n=5). In the entire collection, 61 STs were only identified on a single occasion, representing 28 % of the dataset. In the UK, unique STs represented 23 % of the collection (38 unique STs); whilst in Italy they accounted for 47 % (23 unique STs). Unlike the Italian collection, however, the UK collection was comprised of several replicate isolates from the same animal quarter on different dates. This is discussed further in **Section 3.2.7.1**.

Clonal complex assignment was automatically completed when isolate information was imported into the database; the CC grouping performed by the database places isolates into one of the computer defined CCs if it shares 4 of the seven alleles with the group founder. Using this designation, 91 UK and 4 Italian isolates formed part of the ST 5 CC, 6 UK and 2 Italian isolates were members of the ST 86 CC and 3 UK and 1 Italian isolate were part of the ST 143 CC. The remaining 66 UK and 39 Italian isolates were not part of these complexes but were instead, respectively assigned to 37 and 27 different STs not associated with CCs (according to the PubMLST database).

3.2.6 Observations of recombination

Maximum likelihood (ML) trees were drawn to visualise evolution between mastitis isolates and to allow observations of recombination between the genes. Trees were drawn based upon the nucleotide sequences at each MLST locus. Trees were rendered in TOPALI v.2.5 using PhyML-aLRT (v.2.4.5) with 100 bootstrap runs to provide an idea of the confidence values for resulting figures. Generally the bootstrap values supporting branch groupings were low, suggesting that there is not significant information within the data to make accurate observations of relatedness between most isolates (**Figure 3.7**).

Table 3.2: Allelic profiles of all STs identified from UK and Italian *S. uberis* mastitis isolates.

ST	Locus							CC [A]	Isolates
	arcC	*ddl*	*gki*	*recP*	*tdk*	*tpi*	*yqiL*		
5	1	1	2	1	2	1	2	ST 5	T1-13, T1-26, T1-30, T1-70, T2-5, T2-6, T2-7, T2-15, T2-33, T2-36, T2-44, T2-45, T2-58, T2-61, T3-2, T3-16, T3-18
6	1	1	2	1	2	1	3	ST 5	T1-52, T1-53, T1-73, T2-10, T2-11, T2-12, T2-13, T2-14, T2-20, T2-25, T2-34, T2-38, T2-57, T3-23
10	1	1	2	2	2	1	2	ST 5	T1-71, T2-74
20	1	2	3	2	1	1	6		T2-2, T2-3, T2-27, T2-28, T2-35, T2-37, T2-39, T3-25
22	2	1	2	1	2	1	2	ST 5	T1-68, T3-8
24	2	1	2	2	2	1	2	ST 5	T1-74, T1-75, T2-24, T2-40, T2-43
26	2	3	2	1	2	1	2	ST 5	T2-9, T2-23, T2-56
30	3	1	4	4	3	2	3		I39
35	4	1	2	1	2	1	2	ST 5	T1-24, T1-80, T1-81
67	4	1	2	2	2	1	2	ST 5	T2-42, T2-53, T2-54, T2-55, T2-78
190	6	1	4	2	28	2	3		T3-24
222	5	1	4	3	13	1	3	ST 86	T3-15
233	1	1	2	2	2	1	3	ST 5	T2-63, T2-75
292	5	1	4	3	28	1	3		I1
293	21	30	30	2	44	4	28		I2, I8
294	10	1	5	2	45	4	3	ST 143	I3
295	12	2	5	3	20	1	3	ST 86	I4
296	41	1	5	2	46	4	31		I5
297	10	30	31	1	17	4	33		I7
298	5	1	4	3	28	4	2		I9
299	5	4	4	3	28	4	29		I10, I11
300	39	1	5	2	46	4	32		I12
301	42	2	32	2	47	4	15		I13
302	9	28	3	4	3	1	3		I15
303	2	1	4	2	10	1	3		I16
304	2	1	4	2	49	1	3		I17, I26
305	42	6	5	2	48	14	3		I18, I36, I37, I40, I47
306	2	3	4	2	3	2	3		I19
307	1	1	4	1	2	1	3	ST 5	I20, I48
308	40	1	4	2	49	1	3		I21
309	10	30	3	3	5	1	3	ST 86	I22
310	4	3	29	2	3	1	15		I23
311	10	30	3	3	5	4	3		I24
312	4	1	5	2	2	4	17		I25, I46
313	3	1	5	2	50	1	3		I27
314	2	3	5	1	2	1	3		I28

315	4	31	5	2	51	4	15		I29
316	2	1	4	1	2	1	3	ST 5	I30, I31
317	2	30	4	2	3	4	30		I32
318	42	1	5	2	10	1	3		I35, I38, I45
319	5	15	5	2	2	1	3		I41, I42, I43
320	42	1	5	2	50	4	17		I44
321	2	5	4	2	47	4	10		I50
322	3	28	4	2	3	3	3		T1-3
323	3	4	4	2	3	3	3		T1-4
324	5	1	4	3	58	1	3		T1-5
325	1	33	2	2	5	1	7		T1-6, T1-46, T1-47, T1-48, T1-49, T3-1
326	1	33	2	1	2	1	2	ST 5	T1-9, T1-28, T1-29
327	1	32	2	1	56	2	7		T1-10
328	4	1	9	3	2	1	5		T1-11
329	1	1	2	1	2	1	8	ST 5	T1-18
330	9	23	5	2	55	16	10		T1-20
331	2	1	2	2	3	1	3	ST 143	T1-21, T1-22, T1-23
332	1	1	1	1	2	1	3	ST 5	T1-25, T2-80, T2-81
333	9	2	4	2	57	1	10		T1-27, T3-6
334	4	15	3	2	3	2	3		T1-31
335	4	1	2	2	2	1	3	ST 5	T1-32, T1-33, T1-34, T1-35
336	9	4	5	3	20	3	10		T1-36, T1-37, T1-38, T1-39, T1-40, T1-41, T1-42
337	3	1	4	2	13	17	3		T1-43
338	16	1	3	3	11	3	5		T1-44
339	9	2	5	3	20	3	10		T1-45
340	3	1	11	4	5	3	3		T1-50, T1-51
341	1	1	2	1	56	2	7	ST 5	T1-54, T1-55, T2-76
342	1	3	2	1	2	1	2	ST 5	T1-56
343	5	2	5	2	3	3	3		T1-57, T1-61
344	9	1	4	4	2	1	3		T1-58
345	5	1	4	4	13	3	3		T1-60
346	3	1	35	2	13	1	3	ST 86	T1-62
347	4	1	5	3	3	3	3		T1-63
348	4	23	9	2	13	1	3		T1-64
349	3	1	4	4	3	4	3		T1-65
350	3	2	3	3	3	2	3	ST 86	T1-66
351	3	2	3	2	3	2	3	ST 86	T1-67, T3-5
352	6	34	10	2	28	2	3		T1-69
353	1	1	5	1	2	1	2	ST 5	T1-72
354	1	3	2	2	2	1	2	ST 5	T1-76, T1-77, T1-78, T2-32
355	4	2	3	2	5	4	3		T1-79
356	10	1	5	1	2	1	2	ST 5	T2-1, T2-22, T2-29, T2-46, T3-21

357	1	11	3	2	1	1	6		T2-4
358	1	1	2	1	7	1	2	ST 5	T2-16, T2-17, T2-18, T2-19, T2-30, T2-41,
359	4	1	9	3	42	3	2		T3-32
360	2	1	2	2	2	1	3	ST 5	T2-26
361	2	1	2	1	2	1	3	ST 5	T2-31
362	3	4	3	4	3	1	10		T2-47, T2-49, T3-31
363	1	8	2	1	2	1	3	ST 5	T2-48
364	1	1	2	1	16	1	7	ST 5	T2-50, T2-51, T2-52
365	5	1	5	4	32	1	10		T2-59, T2-60
366	5	1	4	3	20	2	3		T2-62
367	4	1	9	3	2	3	5		T2-66
368	9	23	5	2	55	11	10		T2-67, T2-68, T2-69, T2-70, T2-72
369	4	2	4	3	13	1	3	ST 86	T2-73
370	1	35	2	1	2	1	2	ST 5	T2-79
371	4	4	3	2	42	3	5		T3-3, T3-4
372	3	2	36	4	17	3	3		T3-7
373	21	1	9	3	28	3	5		T3-9
374	9	4	3	4	3	1	10		T3-12
375	16	2	3	4	3	4	12		T3-14
376	9	1	25	4	16	3	10		T3-17
377	21	30	16	2	59	1	28		I6
U 1 [B]	10	1	33	2	53	4	-		I14
U 2 [B]	4	29	34	4	52	15	-		I34
U 3 [B]	4	30	5	2	29	4	-		I49
U 4 [B]	8	2	3	2	3	2	-		T1-59

Alleles and STs were assigned based on comparison to the online database collection. Novel alleles, and thus STs, were assigned at the curator's discretion. Bold STs were novel to the MLST database.

[A] CC assignment based on pre-set database parameters, where a CC is defined as a group of isolates sharing 4 of 7 alleles with the group founder.

[B] Isolates which were not assigned STs due to absence of *yqiL*.

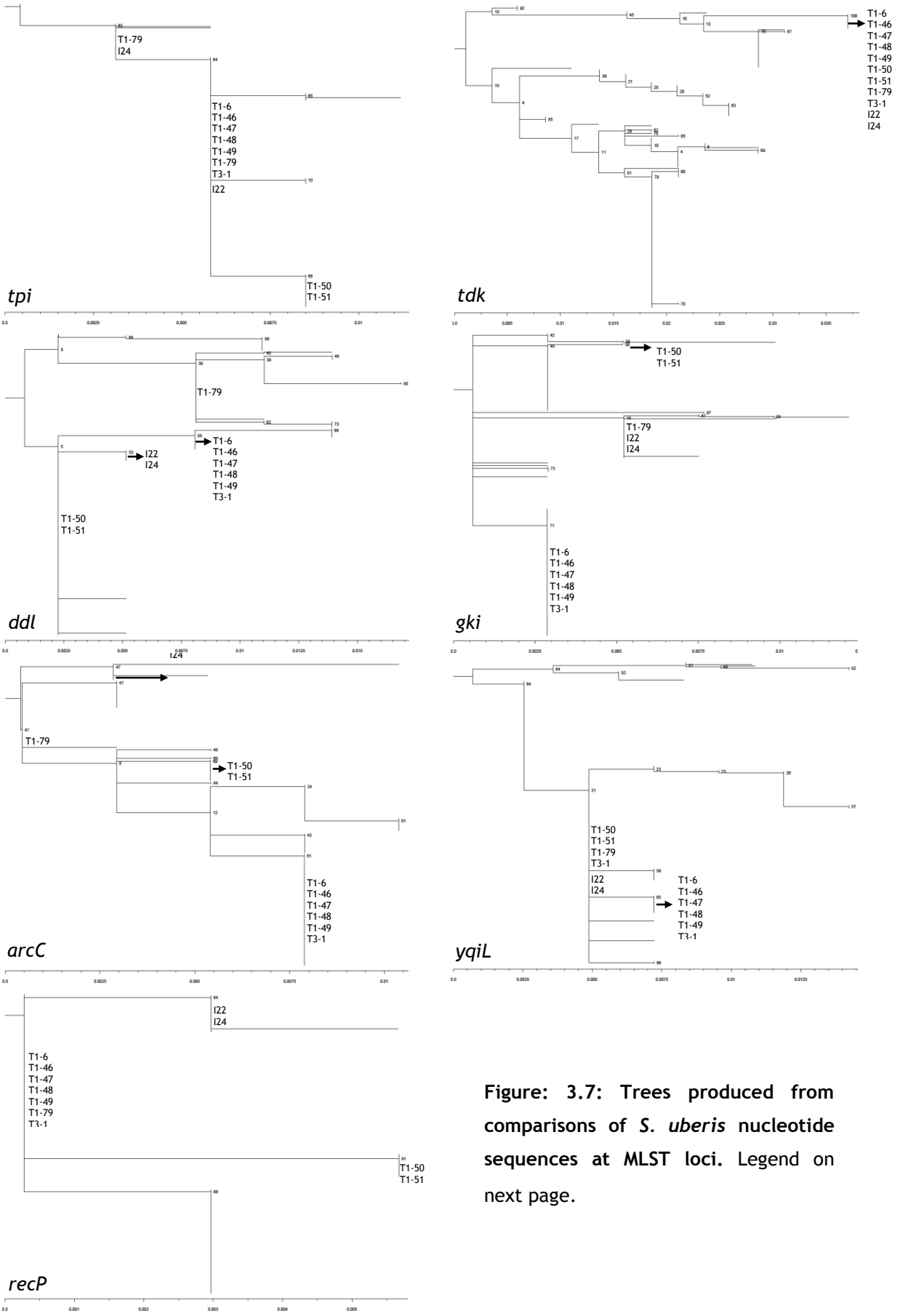


Figure: 3.7: Trees produced from comparisons of *S. uberis* nucleotide sequences at MLST loci. Legend on next page.

Figure 3.7: Trees produced from comparisons of *S. uberis* nucleotide sequences at MLST loci. Nucleotide sequences (in FASTA format) were aligned in TOPALI (Milne *et al.*, 2009) and trees derived using the ML, phyML-aLRT v.2.4.5 algorithm (Anisimova and Gascuel, 2006) and 100 bootstrap runs. To better visualise the resulting tree, the Newick file from TOPALI was loaded into FigTree (v.1.3.1) a tree drawing tool (A. Rambaut, Institute of Evolutionary Biology, University of Edinburgh) and branches were labelled with the bootstrap values. The location of a group of isolates (grouped together with a bootstrap value of 100 % at the *tdk* locus) is shown on all trees to illustrate reticulate evolution between genes.

Differences were observed between the trees, however, and as an example, one group of isolates was chosen to illustrate this observation with the clustering of these isolates on the trees shown in **Figure 3.7**. On the *tdk* tree these isolates were grouped together with high bootstrap support (100 %), but it can clearly be seen that on the additional trees the isolates are not all grouped together. Isolates T1-6, T1-46, T1-47, T1-48, T1-49 and T3-1 remain grouped together on all remaining trees as these share the same ST (ST 325) and isolates T1-46, T1-47, T1-48 and T1-49 all originate from a persistent infection of the same animal quarter. Isolates T1-50 and T1-51 are also replicate isolates from a single persistently infected animal quarter and share the same ST, thus these are grouped together on all trees but are not clustered with any of the other isolates except on the *tdk* and *yqiL* trees. Isolates I22 and I24 are also separated from the other isolates on most of the remaining trees, but are generally clustered together as they share identical alleles (except at the *tpi* locus). Although on the *tdk* tree isolate T1-79 is grouped with all of the other isolates, on two trees this isolate is separate from all isolates and on the remaining trees it is grouped with at least one of the other isolates.

As no tree phylogeny is consistent for all genes, reticulate evolution between genes, or a recombinatorial population structure is implied. This was further demonstrated by the complicated network tree, derived using SplitsTree4 v.4.10 (Huson and Bryant, 2006), based upon the concatenated sequence of all seven housekeeping genes (**Figure 3.8**). The network illustrates reticulate events such as hybridisation or recombination, confirming that the relationship between *S. uberis* isolates cannot be explained by a simple evolutionary model. The frequent recombination evident makes determining common ancestors difficult and considerably more ambiguous; it also confirms that the use of a single tree based on individual or concatenated gene sequences will not display meaningful results as the relationship between isolates will vary at different loci.

3.2.7 Analysis of UK isolates

3.2.7.1 General observations

UK isolates were characterised as persistent or non-persistent based upon the continuation or resolution of mastitis-causing infection following antibiotic treatment. As such, the UK collection contained several isolates which were sequential samples from the same quarter of the same animal on different dates. There were 28 such cases, comprising between two and six isolates from the same animal quarter, implying resistance to antibiotic treatment from the first date, and persistence up to the successive sampling dates. The same ST was identified from both or all replicate

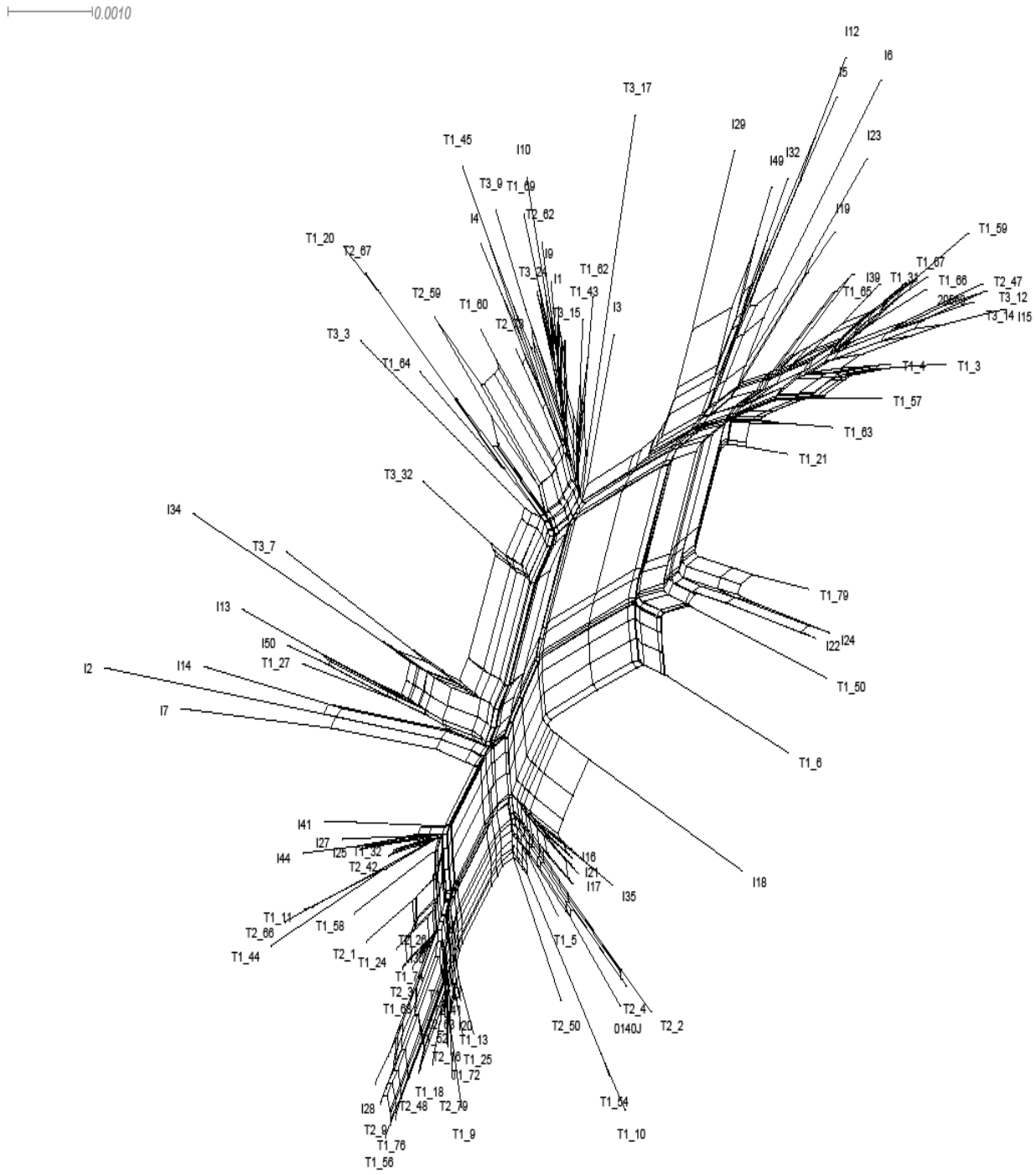


Figure 3.8: A split network representing the diversity amongst *S. uberis* mastitis isolates based upon concatenated sequences from 7 housekeeping genes. To simplify the figure only a single isolate was used to represent each ST, in other words additional isolates with identical sequences at all seven genes are excluded. Sequence variation and phylogenetic relationships were visualised for all isolates by loading concatenated sequences into SplitsTree4 v 4.10 (Huson and Bryant, 2006).

samples of apparently persistent cases on 19 occasions (68 %), suggesting these strains were truly persistent and capable of resisting antibiotic therapy. In the nine cases where the same ST was not identified in replicate samples, eight replicates were assigned different STs, suggesting cure of initial infection and re-infection with a different strain within the time of sampling. In the final case, the same STs were identified from the first two sampling dates, suggesting a persistent infection, however, the third sample was found to be a different ST. Replicate samples from truly persistent cases (n=40) were removed, and a non-redundant list of all remaining UK isolates and corresponding STs are listed in **Table 3.3**. From the 167 samples typed, 127 can thus be considered to be individual infections.

To determine whether successive isolates from a single quarter during a persistent infection, which were determined by MLST to share the same ST, were in fact genetically identical, persistent isolates were analysed by RAPD typing. Initial optimisation was required to determine that primer 5 (GE Healthcare) produced banding patterns which would offer the greatest discriminatory ability. Primer 5 was thus used for RAPD analysis using gDNA from 20 sets of persistent isolates (2 or more isolates per set) from the UK, derived from persistent infections which had been found by MLST to exhibit identical STs (**Figure 3.9**). Results showed that for 16 sets of isolates in which all members displayed the same multi-locus ST, the same RAPD profiles were also seen, offering further evidence that these isolates did indeed derive from a single persistent infection with the same strain. One set of isolates appeared to be from a persistent infection but exhibited distinct multi-locus STs as well as distinct RAPD profiles. In another set, three isolates from the same animal shared the same RAPD profile whilst the fourth displayed a distinct profile despite all four isolates sharing the same ST. Finally, two sets of two isolates which were sampled approx. 1 month apart shared the same multi-locus ST but differed in their RAPD profiles suggesting these were in fact not persistent infections. Interestingly, isolates from different persistent infections but which shared the same multi-locus STs were seen to display different RAPD profiles. Similarly, RAPD analysis of isolates T2-36 (from a non-persistent infection) and T2-5 (from a persistent infection) from different animals on the same farm, both of which were designated ST 5 by MLST, demonstrated that these isolates displayed distinct RAPD profiles.

Twenty three STs were identified more than once from non-replicate samples; most frequently identified STs were ST 5 (n=14), ST 6 (n=9), ST 20 (n=7), ST 24 (n=5) and ST 356 (n=5). Sixteen of the STs represented isolates from more than one farm, with ST 5

Table 3.3: Sequence type and CC designations for UK isolates, excluding replicate samples from persistent infections with the same ST as the original sample.

Isolate ID	Farm	Animal ID	Quarter	ST	DCC/BCC [A]
T1-3	Baker New Ford	187	FL	322 A	-
T1-4	Baker New Ford	187	FL	323 A	-
T1-5	Baker New Ford	289	U	324	-
T1-6	Baker Newton	203	BR	325	-
T1-9	Barton Brook	122	BR	326	ST 5 / 1
T1-10	Bray	5	BL	327	-
T1-11	Britton Sella	140	BL	328	-
T1-13	Bucknell Holways	1847	FL	5	ST 5 / 1
T1-18	Coohe Aller	260	BR	329	ST 5 / 1
T1-20	Crossman Plushayes	60	BL	330	-
T1-21	Crossman Plushayes	75	BR	331 *	ST 143 / 1
T1-24	Crossman Plushayes	108	BR	35 * B	ST 5 / 1
T1-25	Crossman Plushayes	108	BR	332 B	ST 5 / 1
T1-26	Daymond V. Dairy	Whiskey	BL	5	ST 5 / 1
T1-27	Derham Channons	160	FL	333	-
T1-28	Dibble	284	BR	326	ST 5 / 1
T1-29	Dibble	284	FR	326	ST 5 / 1
T1-30	Elston Hotherland	198	FR	5	ST 5 / 1
T1-31	Emmerford	99	FR	334	-
T1-32	Frank Pitt Rix	755	FR	335 *	ST 5 / 1
T1-33	Frank Pitt Rix	755	BR	335*	ST 5 / 1
T1-36	Gale Chevithorne	56	BR	336 *	-
T1-43	Gale Chevithorne	82	FL	337	-
T1-44	Gilbbins H.Hill	473	FL	338	-
T1-45	Gilbbins H.Hill	586	BL	339	-
T1-49	Gilbbins H.Hill	704	FR	325 *	-
T1-51	Hann	19	FR	340 *	-
T1-52	Hann GT. Bradley	2	BL	6 *	ST 5 / 1
T1-54	Hann GT. Bradley	13	FR	341 *	ST 5 / -
T1-56	Harris Southwood	54	FR	342	ST 5 / 1
T1-57	Hayball	Olive	BR	343	-
T1-58	Headon Huntlands	34	BL	344	-
T1-59	Hellier	Camex	FL	Unassigned 4	Not done
T1-60	Hellier Whit	Carmel	BL	345	-
T1-61	Hellier	Jackie	BL	343	-
T1-62	Henson	341	FR	346	ST 86 / -
T1-63	Hill Fulford	2	BR	347	-
T1-64	Hill Fulford	42	BR	348	-
T1-65	Hill Fulford	99	FL	349	-
T1-66	Hill Fulford	125	BR	350	ST 86 / -
T1-67	Hill	68	U	351	ST 86 / -
T1-68	Horrell Bridge	240	BL	22	ST 5 / 1
T1-69	Horrell Bridge	617	U	352	-
T1-70	Hughes - Clares Barn	1	FR	5 C	ST 5 / 1
T1-71	Hughes - Clares Barn	1	FR	10 C	ST 5 / 1
T1-72	Hughes - Clares Barn	31	FL	353 D	ST 5 / 1
T1-73	Hughes - Clares Barn	31	FL	6 D	ST 5 / 1

T1-74	Hughes - Clares Barn	42	BR	24	ST 5 / 1
T1-75	Hughes - Clares Barn	71	BL	24	ST 5 / 1
T1-76	Hughes - Clares Barn	75	BL	354 *	ST 5 / 1
T1-79	Hughes - Clares Barn	127	BL	355	-
T2-1	Hughes - Clares Barn	157	FR	356	ST 5 / 1
T2-2	Hughes - Clares Barn	182	FR	20 *	-
T2-4	Hughes - Clares Barn	182	BR	357	-
T2-5	Hughes - Clares Barn	195	BL	5 *	ST 5 / 1
T2-9	Hughes - Clares Barn	201	FR	26	ST 5 / 1
T2-10	Hughes - Clares Barn	266	FL	6 *	ST 5 / 1
T2-15	Hughes - Clares Barn	279	BR	5	ST 5 / 1
T2-16	Hughes - Clares Barn	293	FL	358 *	ST 5 / 1
T2-20	Hughes - Clares Barn	294	BL	6	ST 5 / 1
T2-22	Hughes - Clares Barn	317	FL	356	ST 5 / 1
T2-23	Hughes - Clares Barn	326	FR	26	ST 5 / 1
T2-24	Hughes - Clares Barn	341	BL	24	ST 5 / 1
T2-25	Hughes - Clares Barn	351	BL	6	ST 5 / 1
T2-26	Hughes - Clares Barn	363	FL	360	ST 5 / 1
T2-27	Hughes - Clares Barn	378	BR	20	-
T2-28	Hughes - Clares Barn	378	FR	20	-
T2-29	Hughes - Clares Barn	400	BL	356 E	ST 5 / 1
T2-30	Hughes - Clares Barn	400	BL	358 E	ST 5 / 1
T2-31	Hughes - Clares Barn	403	FR	361 F	ST 5 / 1
T2-32	Hughes - Clares Barn	403	FR	354 F	ST 5 / 1
T2-33	Hughes - Clares Barn	408	FR	5 G	ST 5 / 1
T2-34	Hughes - Clares Barn	408	FR	6 G	ST 5 / 1
T2-35	Hughes - Clares Barn	445	BR	20	-
T2-36	Hughes - Clares Barn	516	FR	5	ST 5 / 1
T2-37	Hughes - Clares Barn	584	FL	20	-
T2-38	Hughes - Clares Barn	681	BL	6	ST 5 / 1
T2-39	Hughes - Clares Barn	683	FL	20	-
T2-40	Hughes - Clares Barn	728	BL	24	ST 5 / 1
T2-41	Hughes - Clares Barn	728	BR	358 H	ST 5 / 1
T2-42	Hughes - Clares Barn	728	FL	67	ST 5 / 1
T2-43	Hughes - Clares Barn	728	BR	24 H	ST 5 / 1
T2-44	Hughes - Clares Barn	735	BL	5 *	ST 5 / 1
T2-46	Hughes - Clares Barn	750	BR	356	ST 5 / 1
T2-47	Hughes - Clares Barn	753	FL	362 *	-
T2-48	Hughes - Clares Barn	753	BR	363	ST 5 / 1
T2-50	Hughes - Clares Barn	783	BL	364 *	ST 5 / -
T2-53	Hughes - Clares Barn	784	BL	67 *	ST 5 / 1
T2-56	Hughes - Clares Barn	821	BL	26	ST 5 / 1
T2-57	Hughes - Clares Barn	821	FL	6	ST 5 / 1
T2-58	Harris	92	BR	5	ST 5 / 1
T2-59	Kingdom GT. Hayne	1386	FR	365 *	-
T2-61	Lee Dalwood	1091	FL	5	ST 5 / 1
T2-62	Lee Dalwood	99	BR	366	-
T2-63	Lewis Smithincott	160	BR	233	ST 5 / 1
T2-66	Olive LR Coliprest	2	FL	367	-
T2-67	Paine Easteridge	198	BL	368 *	-
T2-72	Paine Easteridge	915	FR	368 I	-

T2-73	Paine Easteridge	915	FR	369 I	ST 86 / -
T2-74	Persey	349	U	10	ST 5 / 1
T2-75	Persey Fordmore	86	FL	233	ST 5 / 1
T2-76	Persey Fordmore	154	BL	341	ST 5 / -
T2-78	Persey Fordmore	327	BL	67	ST 5 / 1
T2-79	Persey Langlands	5	FR	370	ST 5 / 1
T2-80	Persey Langlands	402	BR	332	ST 5 / 1
T2-81	Persey Langlands	513	FL	332	ST 5 / 1
T3-1	Persey Park	D72	BL	325	-
T3-2	Persey Park	L945	FR	5	ST 5 / 1
T3-3	Persey Park	L949	BL	371	-
T3-4	Persey Park	R181	FL	371	-
T3-5	Pyle Northill	U	FL	351	ST 86 / -
T3-6	Pyle Treasebeard	63	BL	333	-
T3-7	Reed Dungeons	39	FL	372	-
T3-8	Reed Dungeons	146	BR	22	ST 5 / 1
T3-9	Reed Dungeons	234	BR	373	-
T3-12	Squire Henland	Dilys	BL	374	-
T3-14	Stacey Jurishayes	71	BL	375	-
T3-15	Stacey Jurishayes	136	FL	222	ST 86 / -
T3-16	Summers Wessington	799	BR	5	ST 5 / 1
T3-17	Summers Wessington	897	BL	376	-
T3-18	Thomas Ewings	82	BL	5	ST 5 / 1
T3-21	Thomas Ewings	454	FL	356	ST 5 / 1
T3-23	Thomas HR Brithayes	72	BL	6	ST 5 / 1
T3-24	Vallis Highdown	250	FR	190	-
T3-25	Vallis Highdown	289	FL	20	-
T3-31	Webber Cranklands	459	FR	362	-
T3-32	Whitnage	Carne	FL	359	-

[A] Clonal complex assignment determined according to the MLST database (DCC) or BURST assignment conducted in this study (BCC). For BCC 1, ST 5 was assigned as the group founder with 96 % confidence as determined by bootstrap re-sampling.

* Occasions where more than one isolate was obtained from a single quarter of the same animal on different dates, and where these isolates all shared the same ST, thus denoting a persistent infection. In these cases, all but the original sample (listed) were excluded from subsequent analyses.

A–I Occasions where replicate samples from the same quarter of the same animal on different dates were found to be assigned to different STs (not truly persistent).

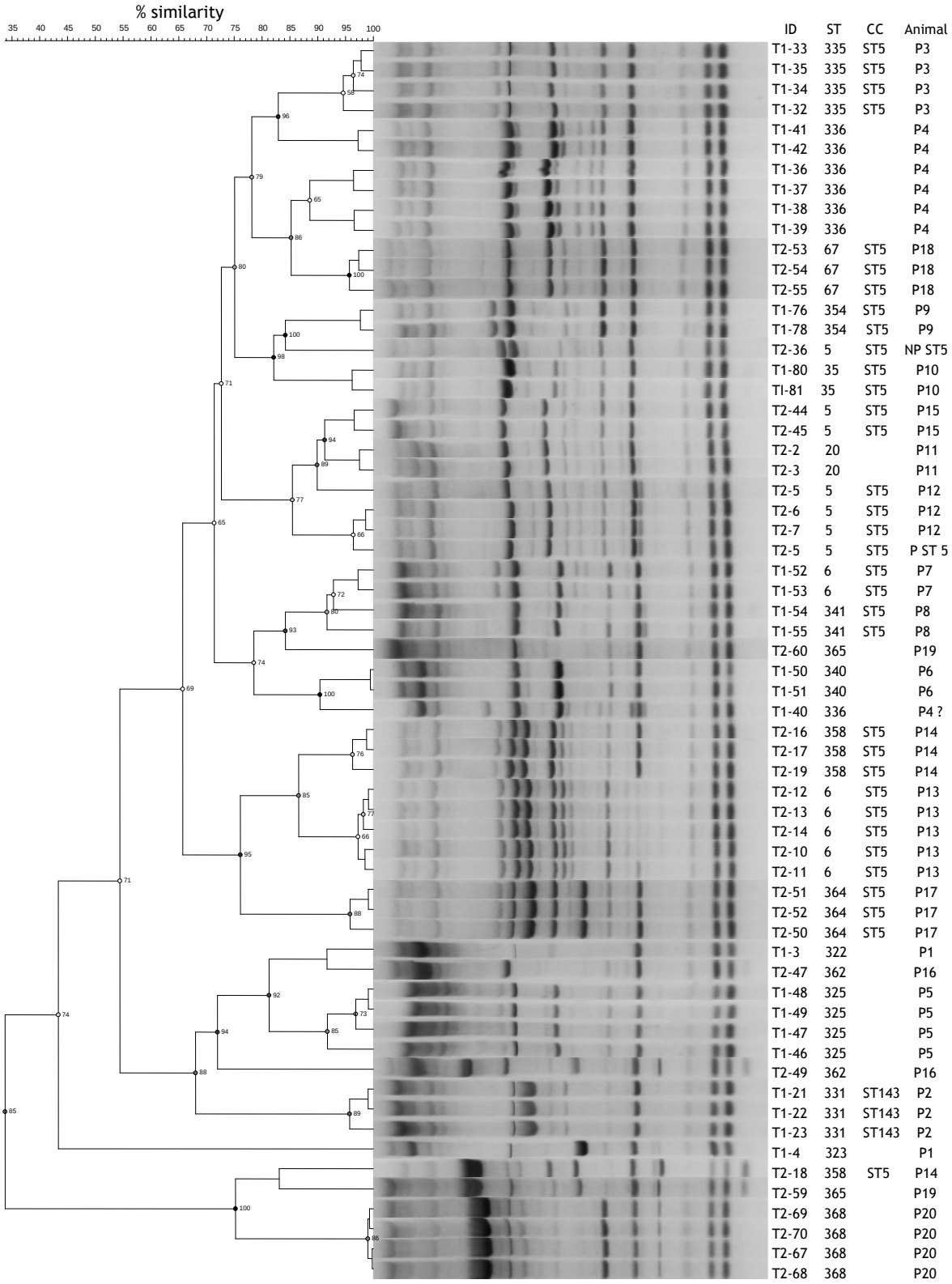


Figure 3.9: Legend on next page

Figure 3.9: Typing of *S. uberis* isolates from putatively persistent mastitis infections using RAPD. In most cases, it was clear that isolates from persistent infections with the same multi-locus ST also shared the same RAPD profile. It was also notable, however, that isolates from different animals which shared the same ST could display distinct RAPD profiles. P1 to P20 identifies the sets of persistent isolates which are from the same animal. Persistent (P ST 5) and non-persistent (NP ST 5) ST 5 isolates originating from different animals on the same farm also display considerably different RAPD profiles.

isolates identified from 9 farms and ST 6 isolates on 3 farms. Isolates representing the same ST were derived from more than one animal on the same farm in 12 cases. There were 3 cases where one animal was infected with isolates sharing the same ST in two different quarters at the same time and in one of these cases, infection persisted in both quarters after initial sampling. There was one case where an animal was infected with isolates sharing the same ST in 2 different quarters on different dates and 2 cases where 2 quarters were infected with 2 isolates with different STs on the same date. There was also evidence of infection spreading from one quarter to the next after initial treatment, as the same ST was identified in isolates from both quarters.

Ten STs, identified more than once, were isolated from both persistent and non-persistent infections, with STs 5, 6 and 20 identified from both persistent and non-persistent infections at equivalent rates considering the sample sizes. Twelve STs were isolated from non-persistent infections only. Notably, ST 335 was the only ST (identified more than once) isolated only from persistent infections; however, the two ST 335 isolates were from two quarters of the same animal on the same date and both caused a persistent infection. As this ST was not found on any other farm or in any other animals, despite its persistence it clearly spread no further than the original infected animal.

3.2.7.2 BURST analysis

To demonstrate relationships between isolates in the UK collection, BURST analysis was conducted. There was high concordance between the largest group identified using the most stringent analysis (6 of 7 alleles shared by group members) and when defining a group as a collection of isolates sharing 5 of 7 alleles. These groups were also almost completely comprised of isolates also assigned by the database to the ST 5 CC (**Tables 3.3 & 3.4**). Eleven groups of isolates sharing at least 5 of 7 alleles were identified, representing 83 % of the infections; whilst 18 isolates remained ungrouped (**Table 3.4**). Groups 2 to 11 contained between 2 and 5 STs whilst the largest group (group 1) represented 26 STs including all the most frequently identified STs (except ST 20), and accounted for 56 % of the infections. The relationships between the STs identified in BURST group 1 are demonstrated diagrammatically in **Figure 3.10**, with ST 5 clearly identified as the group founder, and ST 360 a relatively unrelated subgroup founder.

The frequency with which isolates from persistent or non-persistent infections were associated with STs and BURST groups is displayed in **Table 3.4**. Groups 2, 3, 4, 6, 7 and 8 were all small groups with 5 isolates or less which all comprised only non-persistent isolates. The remaining groups were mixed, with no group comprising just persistent

Table 3.4: Groups identified by BURST analysis of UK *S. uberis* STs and the frequency with which isolates from persistent (P) or non-persistent (NP) infections were identified.

Group	STs	No. of isolates		Group	STs	No. of isolates	
		P	NP			P	NP
1	5 *	2	12		334	0	1
	6	2	7		**All**	**0**	**4**
	10	0	2	5	20	1	6
	22	0	2		357	0	1
	24	0	5		**All**	**1**	**7**
	26	0	3	6	190	0	1
	35	1	0		352	0	1
	67	1	2		**All**	**0**	**2**
	233	0	2	7	323	0	1
	326	0	3		322	0	1
	327	0	1		**All**	**0**	**2**
	329	0	1	8	346 [A]	0	1
	331 [B]	1	0		337	0	1
	332	0	3		**All**	**0**	**2**
	335	2	0	9	374	0	1
	341	1	1		362	1	1
	342	0	1		**All**	**1**	**2**
	353	0	1	10	339	0	1
	354	1	1		336	1	0
	356	0	5		**All**	**1**	**1**
	358	1	2	11	368	1	1
	360	0	1		330	0	1
	361	0	1		**All**	**1**	**2**
	363	0	1	Singletons	325	1	2
	364	1	0		355	0	1
	370	0	1		349	0	1
	All	**13**	**58**		348	0	1
2	222 [A]	0	1		347	0	1
	324	0	1		376	0	1
	345	0	1		344	0	1
	366	0	1		375	0	1
	369 [A]	0	1		343	0	2
	All	**0**	**5**		372	0	1
3	367	0	1		340	1	0
	328	0	1		371	0	2
	359	0	1		338	0	1
	373	0	1		365	1	0
	All	**0**	**4**		333	0	2
4	351 [A]	0	2		Unassigned	0	1
	350 [A]	0	1		**All**	**3**	**18**

A BURST group was defined as isolates sharing at least 5 identical alleles; predicted group founders are identified with an asterisk.

[A-B] STs grouped by the PubMLST database into CC ST 86 and ST 143 respectively.

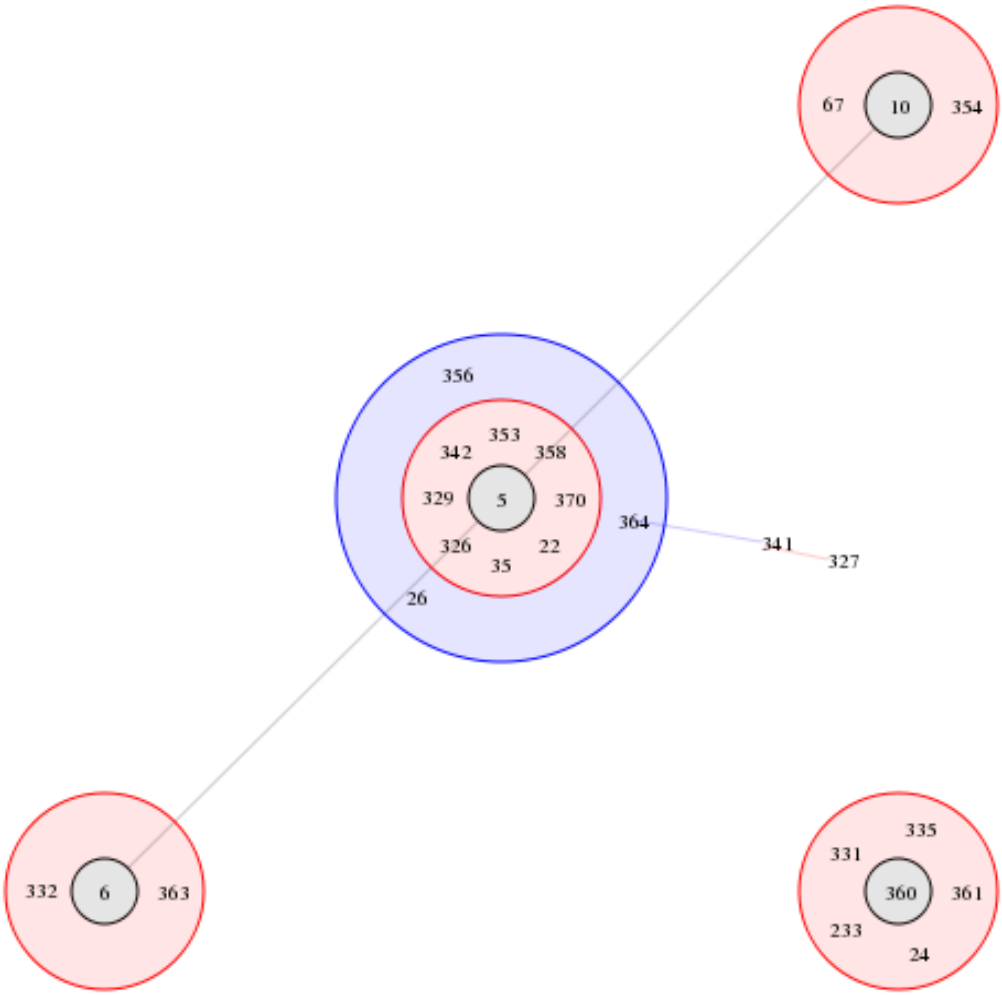


Figure 3.10: Relationships between STs from group 1, as identified and depicted using BURST analysis. A group was defined as a collection of isolates in which at least 5 of the 7 alleles are identical. Central rings denote group or sub-group founders. In this case ST 5 is the group founder, whilst STs 10, 6 and 360 are sub-group founders. Single locus variants (SLVs) are within the first red rings (or separated by a short red line) whilst double locus variants (DLVs) are within the second blue ring (or separated by a longer blue line). Sequence types 10 and 6 are SLVs of ST 5, whilst ST 360 differs from ST 5 at three loci.

isolates. Isolates assigned to the largest BURST group, or the ST 5 CC, were from both persistent and non-persistent infections although most of the persistent cases were found within this group. Notably, all isolates assigned to the ST 86 CC were from non-persistent isolates (n=6). The single isolate assigned to the ST 143 CC was from a persistent infection. Due to small sample sizes, these associations were not, however, statistically significant.

3.2.7.3 Single herd analysis

As 37 % of non-replicate isolates were isolated from a single UK farm, it was possible to analyse the strain variation within a single herd; in this collection of 47 isolates, 18 STs were identified. Sequence types 5, 6, 20, 24, 26, 356 and 358 were found frequently on this farm accounting for 70 % of isolates. Sequence types 24, 26 and 358 were not isolated from any other UK farm (in this study), but whilst ST 358 was unique to this farm the other two STs were isolated several times elsewhere in the UK, as shown on the PubMLST database. Sequence types 5, 6 and 20 were isolated from infections of 5 to 7 animals with each ST responsible for at least one persistent infection. As mentioned previously, two isolates from this farm defined as ST 5; one derived from a persistent infection (T2-5) and one from a non-persistent infection (T2-36) were analysed using RAPD typing and were found to exhibit distinct profiles. The most stringent eBURST analysis demonstrated that most isolates from this farm formed a single CC (**Table 3.5**). When the analysis stringency was lowered, by defining a group as a collection of isolates sharing at least 5 alleles, STs 26 and 364 became part of the main group. Thus, on the basis of MLST analysis, 14 of 18 STs (81 % of isolates) on this farm were defined as genetically closely related.

The second eBURST group contained two STs. Sequence type 20 was isolated frequently (n=6) on this farm and also once on another farm from this study and has been identified frequently in the UK from 1996 to 2002, as shown by PubMLST database entries. In contrast, ST 357 was isolated only once in this study and the ST was novel to the database. Sequence type 357 was identified in an animal where a different quarter had been persistently infected with ST 20 (the last isolate of which was dated from two weeks prior to the isolation of ST 357 in a different quarter). Interestingly, ST 357 is a single locus variant of ST 20 at the *ddl* allele. There were 2 synonymous nucleotide differences between the *ddl* alleles of ST 20 and 357, at positions 176 and 248, where G was exchanged for C and A for C respectively, due either to two point mutations or one recombination event. As genes encoding a penicillin binding protein and superoxide dismutase are located close to the *ddl* gene in strain 0140J, recombination to alter

Table 3.5: Analysis of STs identified on the largest farm studied using eBURST algorithm.

ST	F [A]	SLV	DLV	TLV	SAT	Av. Dist [B]	ST Bootstrap		No. Animals	P [C]	NP [D]
							Group	Subgroup			
Group 1											
5	6	4	6	1	0	1.72	59 %	49 %	6	2	4
10	1	4	4	3	0	1.90	43 %	53 %	1	0	1
6	7	3	4	4	0	2.09	20 %	20 %	7	1	6
24*	5	3	3	4	1	2.27	14 %	15 %	4	0	5
67	2	2	3	5	1	2.45	0 %	0 %	2	1	1
353*	1	2	3	5	1	2.45	1 %	0 %	1	0	1
361*	1	2	3	5	1	2.45	3 %	0 %	1	0	1
360*	1	2	3	3	3	2.63	3 %	0 %	1	0	1
358*	3	1	3	6	1	2.63	0 %	0 %	3	1	2
354*	2	1	3	5	2	2.72	0 %	0 %	2	1	1
363*	1	1	2	5	3	2.90	0 %	0 %	1	0	1
356	4	1	1	6	3	3.0	0 %	0 %	4	0	4
Group 2											
20	6	1	0	0	0	1.0	-	-	5	1	5
357*	1	1	0	0	0	1.0	-	-	1	0	1
Singletons											
355*	1	-	-	-	-	-	-	-	1	0	1
364*	1	-	-	-	-	-	-	-	1	1	0
362	1	-	-	-	-	-	-	-	1	1	0
26*	3	-	-	-	-	-	-	-	3	0	3

The number of re-samplings used for bootstrapping was 1,000 and a group was defined as isolates which share 6 alleles with at least one other group member. Group founder predictions were calculated by the software based upon the number of SLVs, DLVs, TLVs and satellite variants (SAT) which are more distantly related. Although identified as singletons in this analysis, in a subsequent analysis, ST 364 and ST 26 form part of the ST 5 CC.

[A] Frequency (F) with which ST was found in this collection from the same farm (not including replicate samples from the same animal)

[B] Average distance refers to the average number of locus differences between allelic profiles.

[C] Number of isolates from persistent infections.

[D] Number of isolates from non-persistent infections.

* ST identified on this farm only (out of the total UK collection analysed in this study).

these genes may have driven the change of the ST 20 *ddl* allele, leading to the generation of a new ST.

3.2.8 Analysis of Italian isolates

3.2.8.1 General observations

The Italian collection comprised 49 isolates from ovine or bovine mastitis cases from different farms in the Lazio region of Italy. In the ovine collection, 13 isolates (the origin of one isolate was unknown), were collected from eight farms in two regions, whilst in the bovine collection twenty one farms and five regions were represented. The 49 isolates were resolved into 32 STs (**Table 3.6**), with three isolates remaining unassigned (1 bovine, 2 ovine) as the *yqiL* gene region could not be amplified from these isolates. Thirty one of the STs identified were novel; the remaining ST had been identified previously in two UK bovine isolates. This was the only ST in this collection that was shared between isolates in two countries.

Of the 32 STs, only nine were represented by more than one isolate; of these, five were represented by two or three isolates from the same farm only. Three STs comprised isolates from different farms in the same region and just one ST contained two isolates from different regions. No STs represented both ovine and bovine isolates, and in fact the fourteen ovine isolates were resolved into 11 different STs, one isolate was unassigned and just two STs were each represented by two isolates, both of which were from the same farm. The diversity among the ovine isolates thus appears to be higher than among the bovine isolates, despite equivocal numbers of farms and regions being represented in comparison to group size.

3.2.8.2 BURST analysis

Stringent BURST analysis, defining a group (or CC) as a collection of isolates sharing at least 6 alleles, identified a single group of just 4 isolates (**Table 3.6**). The stringency of analysis was lowered so that a group was defined as a collection of isolates sharing at least five alleles, using these parameters five groups were identified within the Italian collection (**Table 3.7**). Groups 2, 4 and 5 were represented by 2 STs and by just two or three isolates; Group 1 by 3 STs and four isolates, whilst the largest group was represented by 8 STs and 13 isolates and accounted for 26.5 % of the collection (**Figure 3.11**). Groupings obtained by BURST analysis of allelic profiles were highly correlated to the clusters visualised on a phylogenetic tree, produced using 'web tools' on the MLST database (**See Figure 4.13, Chapter 4**). The remaining 22 isolates (7 ovine and 15

Table 3.6: Sequence types and CCs identified amongst Italian *S. uberis* mastitis isolates.

ST	Isolate (s)	Origin [A]	Source [B]	DCC/BCC [C]	*pauA*	*gapC*
30	I39	B	Savone, RM		9	4
292	I1	O	Cricchi Valerio, RM		9	7
293	I2, I8	O	Manca, VT		Absent	30
294	I3	O	Zappaterreno, RM	ST 143	Absent	32
295	I4	O	Eraldo, RM	ST 86	1	7
296	I5	O	Rossetti, RM		1	33
297	I7	O	Unknown		Absent	33
298	I9	O	Manca, VT		9	7
299	I10, I11	O	Cricchi Valerio, RM		9	7
300	I12	O	Tagliaferri, RM *		Absent	33
301	I13	O	Miani, RM *		1	27
302	I15	B	CRA, RM		1	12
303	I16	B	Ascenzi, VT	1	23	4
304	I17	B	Marini, RM	1	23	4
304	I26	B	Greci, RM	1	23	4
305	I18	B	Coculo, RM		6	29
305	I36, I40, I47	B	Colognesi, RM		6	29
305	I37	B	Cinque Stelle, RM		6	29
306	I19	B	Casilina, RM		23	19
307	I20	B	Fabi, FR	ST 5	4	8
307	I48	B	Coculo, RM	ST 5	4	8
308	I21	B	CRA, RM	1	6	8
309	I22	B	Marini A.M., VT	ST 86	9	7
310	I23	B	Marini A.M., VT		Absent	1
311	I24	B	Marini A.M., VT		9	34
312	I25, I46	B	D'Angelo, RM		6	8
313	I27	B	Santini, RM		6	8
314	I28	B	Santini, RM		4	21
315	I29	B	Zelli, RI		Absent	1
316	I30, I31	B	Paniccia, RM	ST 5	23	4
317	I32	B	Marocca, FR		Absent	31
318	I35, I38	B	Colognesi, RM		4	4
318	I45	B	Maggi, RM		4	4
319	I41, I42, I43	B	Cremona, RM		9	1
320	I44	B	Paganelli, LT		6	4
321	I50	B	Buglione, LT *		33	31
377	I6	O	Ferretti, RM		32	3
Unassigned 1	I14	O	Tagliaferri, RM *		1	28
Unassigned 2	I34	B	CRA, RM		1	3
Unassigned 3	I49	B	Zuchi, VT *		1	1

Isolates were provided by F. Cancellotti, Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana (IZSLT).

[A] Host species was either bovine (B) or ovine (O)

[B] Farm ID and region of Lazio, Roma (RM), Viterbo (VT), Frosinone (FR), Rieti (RI) and Latina (LT) from which isolates originated. Asterisk denotes isolates were sampled in 2007 (remainder 2006).

[C] Database assigned CCs (DCC) and BURST assigned CCs (BCC) based on isolates sharing at least 6 of 7 alleles.

Table 3.7: Groups of closely related Italian *S. uberis* isolates identified using BURST analysis.

Group	ST	Isolate(s)	Source	*pauA* allele	*gapC* allele	Group differences using *gapC* not *yqiL*
1	292	I1	Ovine	9	7	
	298	I9	Ovine	9	7	None - as previously
	299	I10, I11	Ovine	9	7	
2	296	I5	Ovine	1	33	None - as previously
	300	I12	Ovine	Absent	33	
3	303	I16	Bovine	23	4	
	304*	I17, I26	Bovine	23	4	
	307	I20, I48	Bovine	4	8	As previously, except I27 and I28 are absent and replaced with I44
	308	I21	Bovine	6	8	
	313	I27	Bovine	6	8	
	314	I28	Bovine	4	21	
	316	I30, I31	Bovine	23	4	
	318	I35, I38, I45	Bovine	4	4	
4	309	I22	Bovine	9	7	None - as previously
	311	I24	Bovine	9	34	
5	312	I25, I46	Bovine	6	8	Group 5 now comprises isolates I32 and I50
	320	I44	Bovine	6	4	
						Additional group (6) comprises I29 and I49
Singletons	293	I2, I8	Ovine	Absent	30	
	294	I3	Ovine	Absent	32	
	295	I4	Ovine	1	7	
	297	I7	Ovine	Absent	33	
	301	I13	Ovine	1	27	
	302	I15	Bovine	1	12	As previously except isolates I29, I32 and I50 are absent, whilst isolates I14, I34, I27, I25, I46 and I28 are included
	305	I18, I36, I40, I47, I37	Bovine	6	29	
	306	I19	Bovine	23	19	
	310	I23	Bovine	Absent	1	
	315	I29	Bovine	Absent	1	
	317	I32	Bovine	Absent	31	
	30	I39	Bovine	9	4	
	319	I41, I42, I43	Bovine	9	1	
	321	I50	Bovine	33	31	
	377	I6	Ovine	32	3	

A group was defined as a collection of isolates sharing at least 5 of 7 alleles. The differences in BURST groupings when *gapC* was used instead of *yqiL* are listed in the final column and compared to the isolates listed in the third column.

* ST 304 is assigned as the predicted group 3 founder based on the number of SLV, DLV and TLVs.

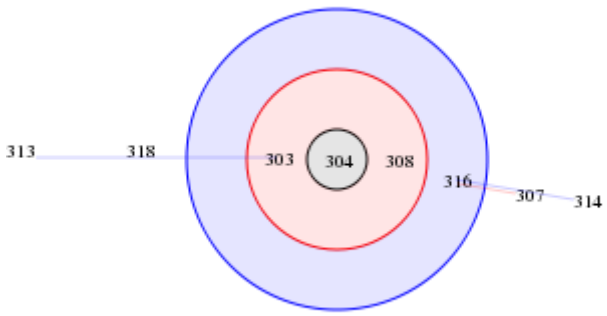


Figure 3.11: Relationships between STs belonging to group 1 as identified by BURST analysis of allelic profiles from Italian isolates. The central ring denotes the group founder whilst STs within the first red ring are SLVs of the group founder. The STs within the second blue ring are DLVs of the group founder. Short red lines also connect SLVs and longer blue lines connect DLVs.

bovine) representing 15 STs were not placed into groups as they were sufficiently divergent from all other isolates. Interestingly, 7 of the 8 isolates identified as *pauB*-positive were in this group of singletons, suggesting significant genetic diversity between these isolates. Groups 1 and 2 were represented by only ovine isolates, whilst only bovine isolates formed groups 3, 4 and 5, demonstrating the heterogeneity between the populations derived from the two host species. One group, comprised isolates from one farm only, whilst all other groups were represented by isolates from at least two different farms. Of these, one group was represented by isolates from the same region only, whilst the three remaining groups were represented by isolates from two or three different regions. This may suggest that whilst diversity among this collection is generally high, certain STs, or groups of STs are more prevalent and appear to be spread throughout the area, not limited to individual farms.

3.2.8.3 *pauA* and *gapC* alleles

To determine the effect of including virulence genes in the established MLST scheme, PCR products derived from amplification of *pauA* and *gapC* were sequenced (it should be noted that *gapC* is in fact considered to have multiple functions as both a housekeeping and a virulence gene). Due to time and cost restraints this was only done for the Italian collection. Allele numbers were assigned following comparison to sequence data previously generated as part of the published study by Zadoks, *et al* (2005a), with the assistance of R. Zadoks (Division of Epidemiology and Population Biology, Moredun Research Institute). The *pauA* and *gapC* alleles identified are listed in **Table 3.6**. As the *pauA* gene was absent from over 10 % of the Italian *S. uberis* collection, like *yqiL*, it is a poor MLST target. With the exception of 2 novel *pauA* alleles identified in the course of this study, the remaining 5 had been previously described in strains from other countries, suggesting low diversity within this allele (despite a high rate of non-synonymous changes resulting in a high dN/dS ratio and indicating strong selection pressure on this region). The *pauA* alleles identified in all isolates belonging to BURST groups 1, 4 and 5 were identical within the groups, whilst most *pauA*-negative isolates were defined as singletons (**Table 3.7)**. No obvious difference was seen between *pauA* alleles of isolates from sheep or cows, except increased absence of *pauA* in sheep.

Alternatively, the multifunctional *gapC* gene was found in all isolates and half of the *gapC* alleles identified in this study were novel, with 16 alleles found from the 49 Italian isolates (8 ovine and 8 bovine). Only two *gapC* alleles were shared between ovine and bovine isolates, with allele 7 predominating in the ovine population whilst present in

just one bovine isolate. Allele 3 was found only once in both ovine and bovine collections, but alleles 4, 7, 8 and 33 appeared to be associated mostly with isolates from specific BURST groups. Significantly, both the *pauA* and *gapC* alleles were the same in all isolates sharing the same STs.

3.2.8.4 eBURST analysis using *yqiL* or *gapC* as the seventh MLST target

eBURST analysis was conducted using alleles from either the *gapC* or *yqiL* loci as the seventh MLST target and including previously excluded isolates I14, I34 and I49 which were assigned an arbitrary allele number at the *yqiL* locus and thus given unique STs to permit analysis. Analysis by eBURST using the *yqiL* alleles showed no change from the original analysis except that the previously unassigned isolates were now also assigned as singletons. The eBURST groups identified when *gapC* was utilised instead are shown in **Table 3.7**. A high similarity was seen between the different analyses, and an equal number of isolates formed groups or were designated as singletons. Notably, however, when *gapC* alleles were used for analysis, I49, a previously unassigned isolate, formed part of a small additional group. Amplification of *gapC* was also achieved from all 167 *S. uberis* mastitis isolates from the UK collection studied, demonstrating that unlike *yqiL*, *gapC* appears to be conserved amongst *S. uberis* isolates; this being consistent with previous observations that deletion of *gapC* homologues are lethal to pathogenic streptococci (Gase *et al.*, 1996; Winram and Lottenberg, 1998).

3.2.9 Comparison of UK and Italian isolates

The strain diversity within the UK and Italian collections was analysed, in addition to that amongst the persistent and non-persistent and the ovine and bovine sub-collections. This was firstly calculated simply by dividing the number of isolates in the collection by the number of STs identified in the collection (**Table 3.8**). The index of association and Diversity index for the collections were further calculated using the Standardised (Haubold) method (calculated by START2) and Simpsons index of diversity, respectively. A greater index of association was seen amongst UK over Italian isolates, but in both collections the observed variance was greater than the maximum trial variance (and thus greater than 0). This suggested linkage disequilibrium, and thus a clonal population at all levels, with recombination being limited (in contrast to the results obtained by Splits tree analysis). Diversity indexes obtained for UK and Italian collections were, however, very similar and indicated that two randomly-picked isolates would in almost 98 % of times fall into two different STs. Diversity index calculations further demonstrated the greater heterogeneity between ovine and persistent isolates

Table 3.8: Diversity calculations for *S. uberis* collections and sub-populations studied.

Collection	No. of STs	No. of isolates	Mean isolates per ST	DI [A]	I^S_A [B]	LD [C]
UK collection						
Persistent	17	20	1.18	0.984	0.230	+
Non-Persistent	60 & 1 Unassigned	107	1.78	0.975	0.263	+
All UK	67 & 1 Unassigned	127	1.90	0.975	0.256	+
Italian collection						
Ovine	11 & 1 Unassigned	14	1.27	0.974	0.177	+
Bovine	21 & 2 Unassigned	35	1.67	0.962	0.131	+
All Italian	32 & 3 Unassigned	49	1.53	0.979	0.107	+
All isolates	99	176	1.78	0.982	0.194	+
DATABASE	392	857	2.19	ND	ND	ND

Persistent isolates were more heterogeneous than non-persistent isolates, whilst Italian ovine isolates were more heterogeneous than Italian bovine isolates.

[A] Discriminatory index (DI) was calculated using Simpson's index of diversity (Hunter and Gaston, 1988).

[B] Standardised index of association (I^S_A) (Smith *et al.*, 1993) was calculated using START2. In all cases I^S_A values were significantly greater than zero and the observed variance was greater than the maximum variance in 1000 trials (P= 0.000), thus all collections may be considered to be clonal.

[C] Linkage disequilibrium (LD) was detected (+) within a population as evidenced by an I^S_A value of greater than zero.

ND Not done.

over bovine or non-persistent isolates (**Table 3.8**).

All UK and Italian isolates were subjected to BURST analysis, with the group definition set as a collection of isolates sharing at least 5 of 7 alleles. Groups of STs identified are shown in **Table 3.9**. Many Italian STs unexpectedly formed groups with UK STs, especially notable in groups 1 and 5 (**Figure 3.12**) which incorporated bovine or ovine Italian STs respectively. Most interestingly, all isolates from the largest group identified in each country by earlier BURST analyses were found within BURST group 1 in this analysis. Whilst nearly all Italian and UK STs are distinct, this result clearly demonstrates that these STs are actually genetically similar and represent the strains observed most frequently in both countries.

3.2.10 Comparison of isolates to the database collection

At the time of writing, 857 isolates had been submitted to the *S. uberis* MLST database (http://pubmlst.org/suberis/), including those submitted for this study. The majority of isolates were from the UK (n=412) and New Zealand (n=254), with just 16 % from 6 other European countries. The percentage of isolates from cattle was 86 %, with just 1.63 % from sheep (predominantly provided by this study), 0.12 % from buffalo and 11.79 % from the environment (0.46 % unknown).

Conducting a population snapshot of the entire database collection can be completed using the least stringent eBURST settings where in a group no alleles need to be identical. This allows the global relationship between all STs to be visualised, and specific populations highlighted to demonstrate where they lie within the total *S. uberis* population (**Figures 3.13 and 3.14**). These images show that many of the UK STs from this study are part of the central complex, which actually includes the ST 5 and ST 143 CCs, suggesting that these two large CC's are also genetically similar and have common ancestry. Italian isolates were not generally associated with this complex, although it has been shown in this study that there is similarity between some Italian and UK STs. Clearly this population snapshot does not accurately identify all relationships, as, for example, STs 303 and 304 are shown as outliers yet in fact they share 5 of 7 alleles with ST 316 which is part of the central complex. Under the parameters of the programme, isolates not forming part of the central complex are visualised as outliers with few groups formed between them (and their relative positioning is not meaningful). However, small groups, for example the one comprising ST 20 (which was frequently found in the UK collection) as the founder, do appear to be emerging, along with the ST

Table 3.9: BURST groups identified by analysis of STs from UK and Italian collections.

ST	Freq [A]	Source	ST	Freq [A]	Source	ST	Freq [A]	Source
GROUP 1			**GROUP 4**			**Singletons**		
5*	17	UK	190	1	UK	293 [O]	2	Italy
6	14	UK	352	1	UK	294 [O]	1	Italy
10	2	UK	**GROUP 5**			295 [O]	1	Italy
22	2	UK	222	1	UK	297 [O]	1	Italy
24	5	UK	292* [O]	1	Italy	301 [O]	1	Italy
26	3	UK	298 [O]	1	Italy	305	5	Italy
35	4	UK	299 [O]	2	Italy	306	1	Italy
67	5	UK	324	1	UK	310	1	Italy
233	2	UK	345	1	UK	315	1	Italy
303	1	Italy	366	1	UK	317	1	Italy
304	2	Italy	369	1	UK	319	3	Italy
307	2	Italy	**GROUP 6**			321	1	Italy
308	1	Italy	296 [O]	1	Italy	325	6	UK
313	1	Italy	300 [O]	1	Italy	333	2	UK
314	1	Italy	**GROUP 7**			338	1	UK
316	2	Italy	302	1	Italy	340	2	UK
318	3	Italy	362	3	UK	343	2	UK
326	3	UK	374	1	UK	347	1	UK
327	1	UK	**GROUP 8**			348	1	UK
329	1	UK	309	1	Italy	355	1	UK
331	3	UK	311	1	Italy	365	2	UK
332	3	UK	**GROUP 9**			371	2	UK
335	4	UK	312	2	Italy	372	1	UK
337	1	UK	320	1	Italy	375	1	UK
341	3	UK	**GROUP 10**			376	1	UK
342	1	UK	322	2	UK	377	1	Italy
344	1	UK	323	1	UK			
346	1	UK	**GROUP 11**					
353	1	UK	328	1	UK			
354	4	UK	359	1	UK			
356	5	UK	367	1	UK			
358	6	UK	373	1	UK			
360	1	UK	**GROUP 12**					
361	1	UK	330	1	UK			
363	1	UK	368	5	UK			
364	3	UK	**GROUP 13**					
370	1	UK	334	1	UK			
GROUP 2			350	1	UK			
20	8	UK	351	2	UK			
357	1	UK	**GROUP 14**					
GROUP 3			336	7	UK			
30	1	Italy	339	1	UK			
349	1	UK						

* Denotes the predicted group founder for groups of isolates sharing at least 5 of 7 alleles.

[A] Frequency (Freq) with which the ST was identified.

[O] STs representing ovine isolates (all remaining STs represent bovine isolates).

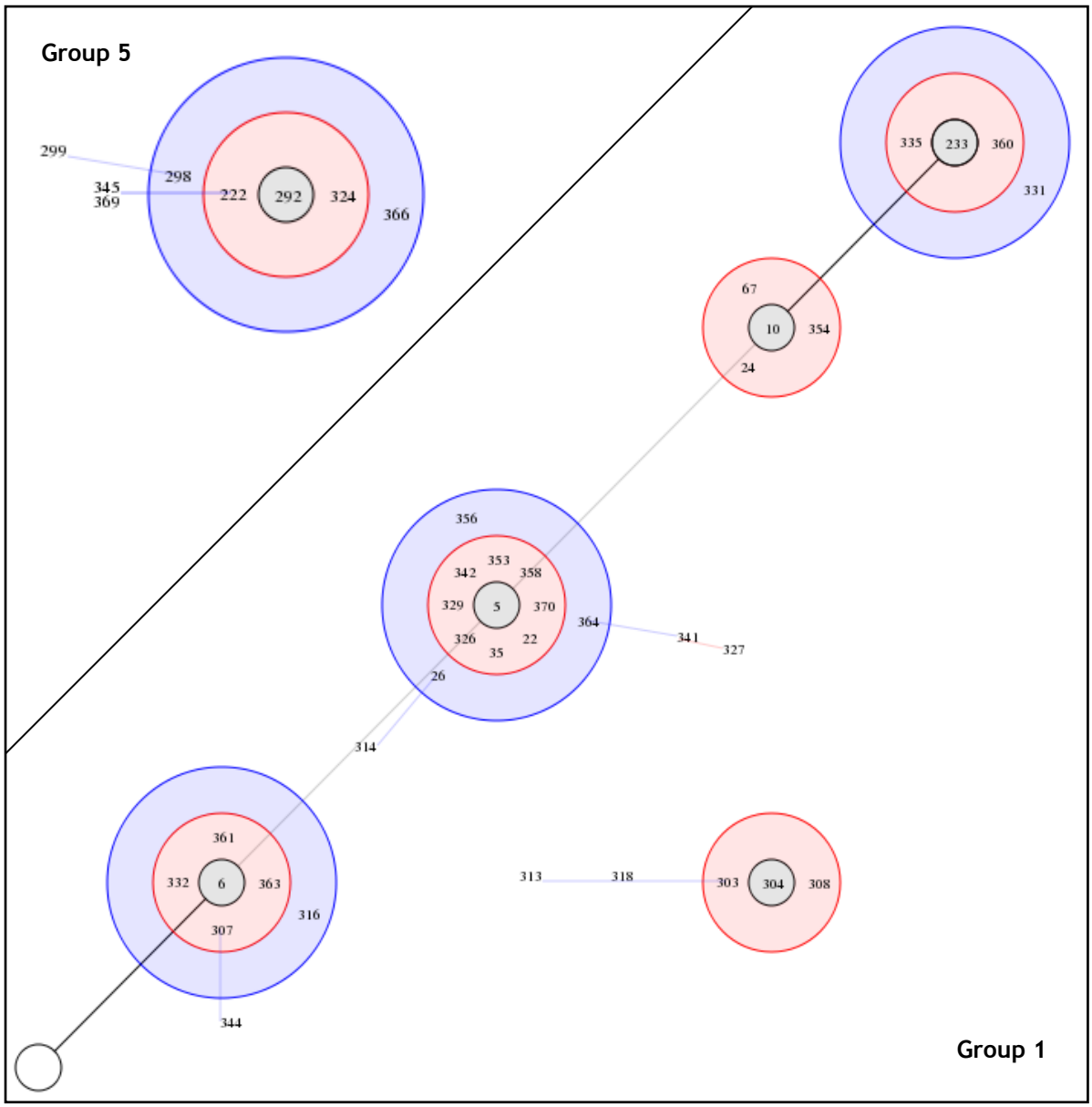


Figure 3.12: Relationships between STs in groups 1 and 5 identified from BURST analysis of allelic profiles from UK and Italian *S. uberis* isolates. A group was defined as a collection of isolates sharing identical alleles at 5 of 7 genes. In group 1 it is notable that STs 304, 303, 308, 313 and 318, which are from Italian bovine cases, form a distinct cluster within this group suggesting no direct ancestry between these isolates. In group 5 several of the Italian ovine STs, ST 292, 298 and 299, unexpectedly form a small group with STs from the UK which are of bovine origin.

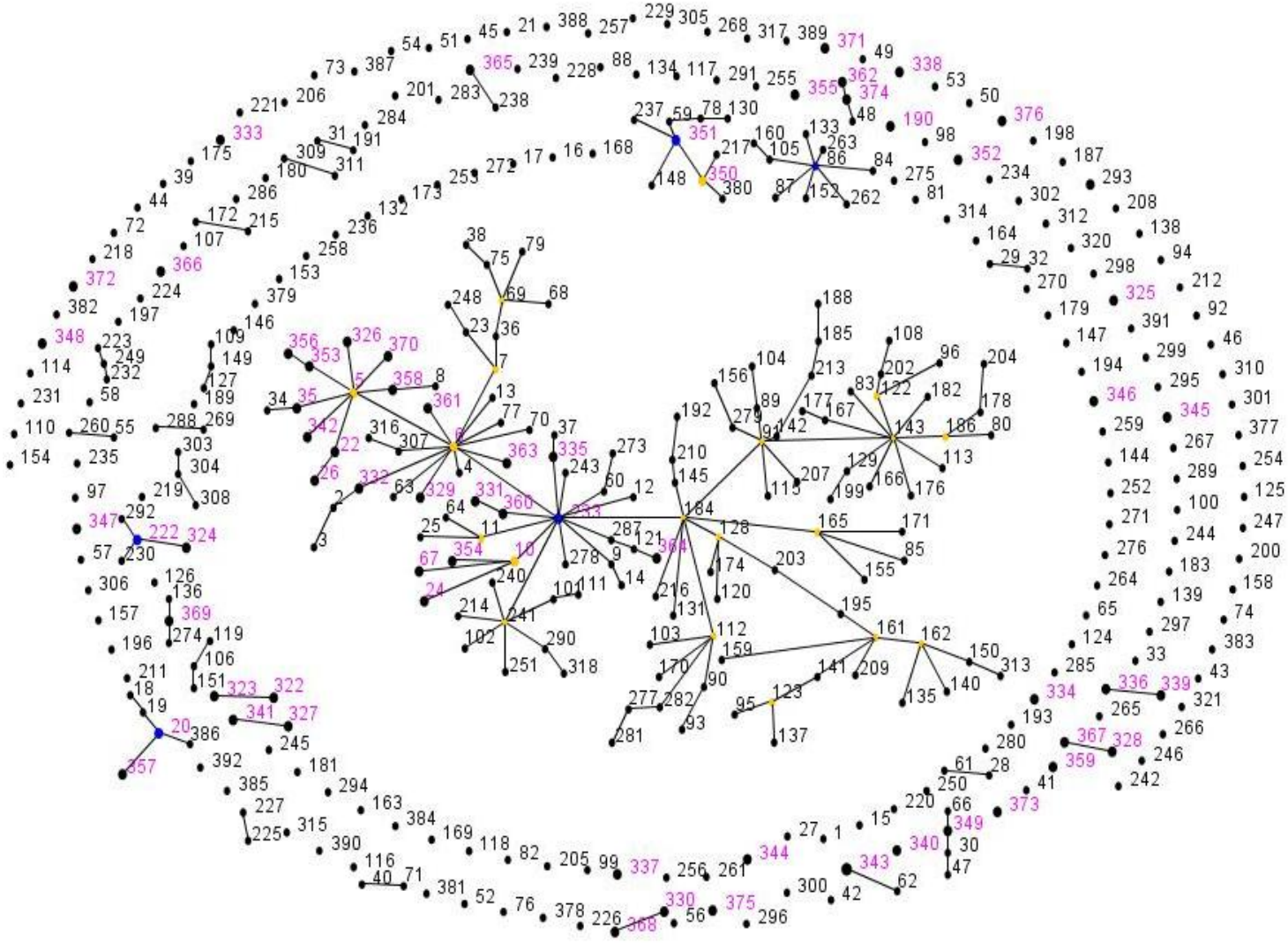


Figure 3.13: Population snapshot of all *S. uberis* STs in the PubMLST database revealing global relationships. By setting the eBURST grouping to zero all STs can be compared directly, in this figure all STs found in the UK collection from this study are highlighted and a large percentage of these STs are found within the central cluster of closely related STs.

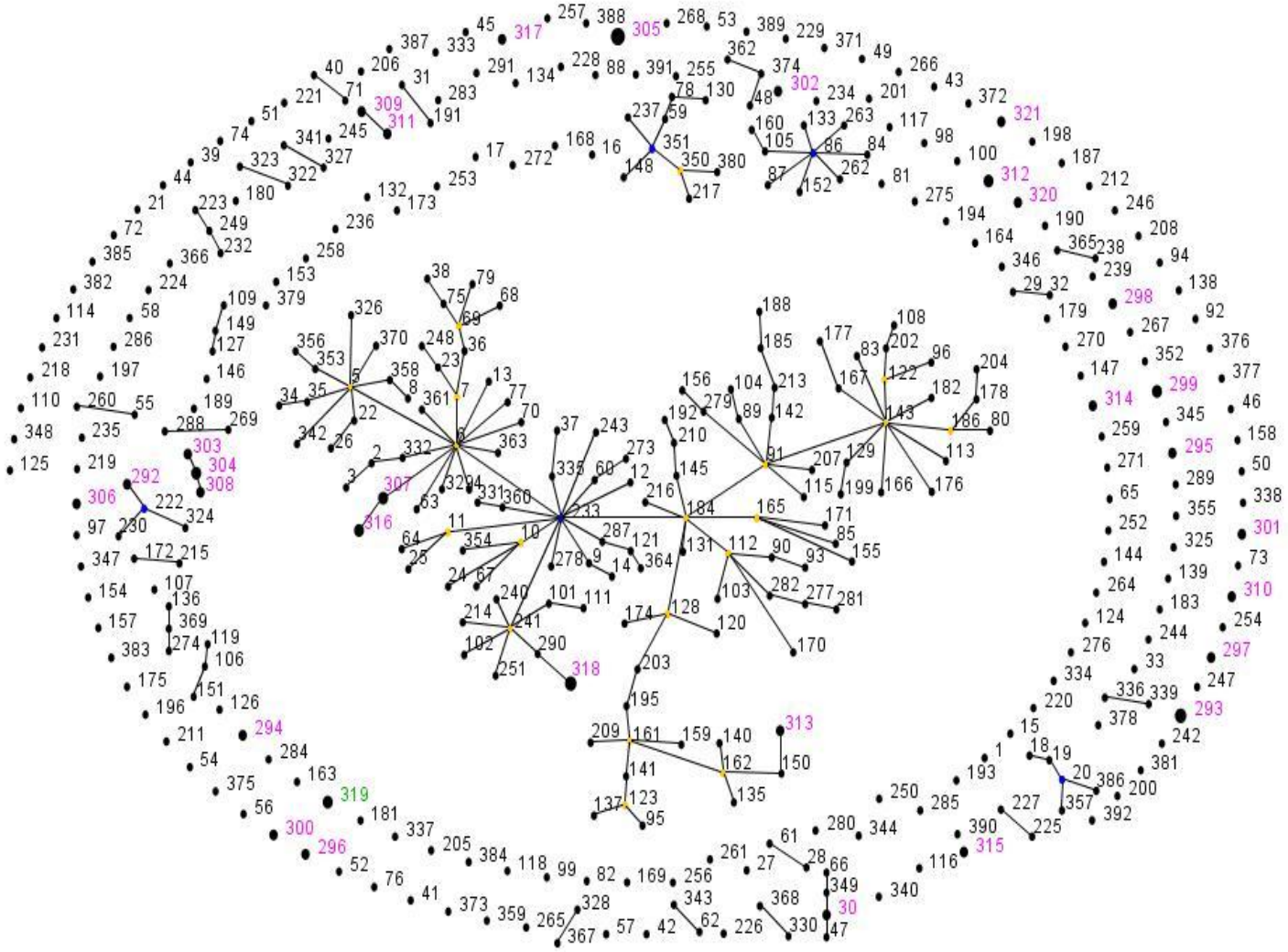


Figure 3.14: Population snapshot of all *S. uberis* STs in the PubMLST database revealing global relationships. By setting the eBURST grouping to zero all STs can be compared directly, in this figure all STs found in the Italian collection from this study are highlighted and they predominantly lie out with the major cluster.

86 CC.

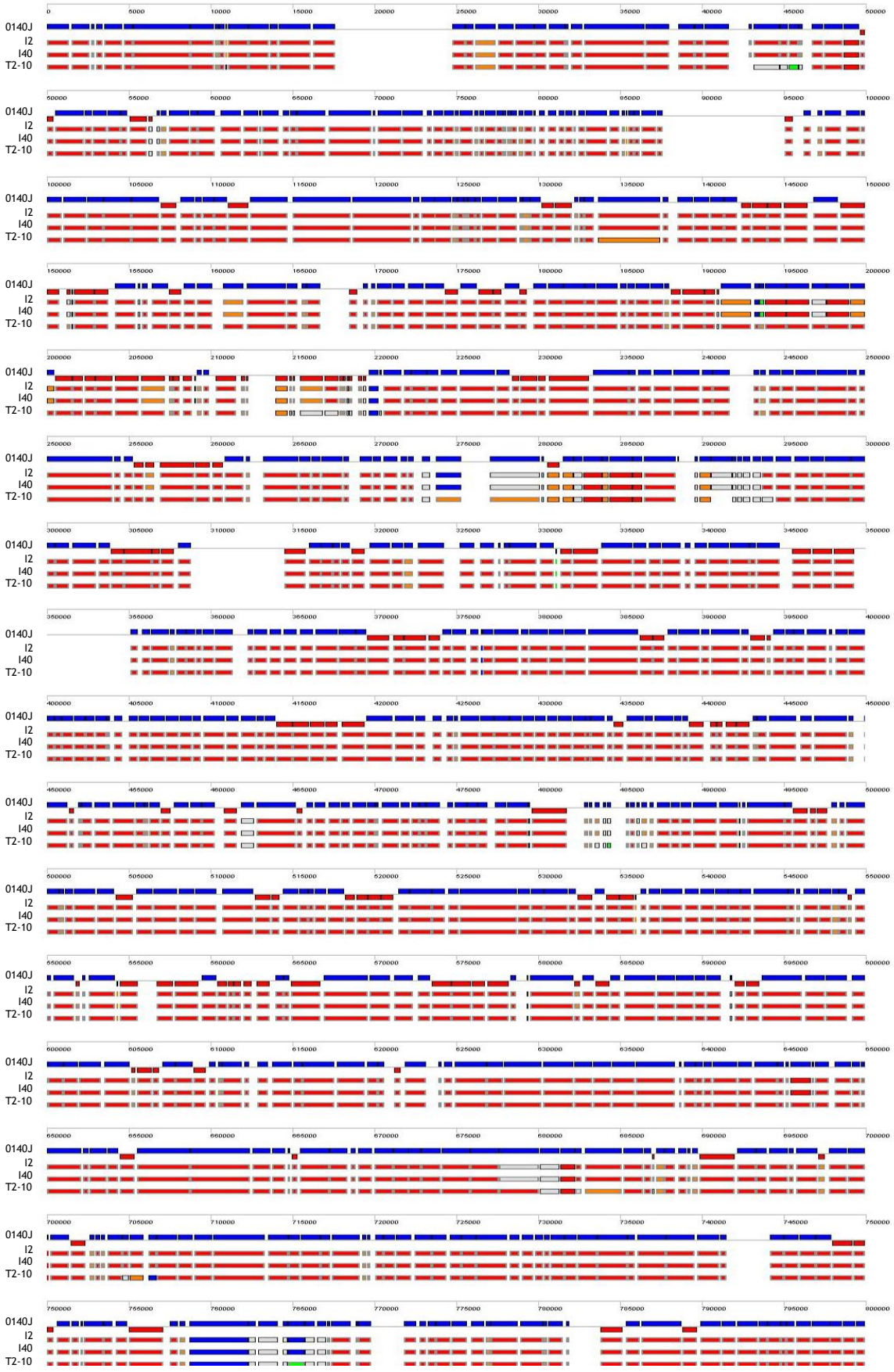
Searching the database, a small set of additional bovine Italian isolates was found to have been submitted after those from this study. Of these 8 isolates, 2 share STs with 5 of the Italian isolates submitted in this study. One of these isolates was typed as ST 304, the founder of the dominant Italian group identified in this chapter. A further novel ST was identified which was related to the dominant group (ST 391), whilst another (ST 390) prompted the formation of an additional 6th BURST group with ST 315 (isolate I29), which was previously ungrouped in this study. These results appear to confirm the discovery of a new Italian CC comprising strains dominant to this region, as has been found in the UK and New Zealand, and all these dominant strains from each country appear to be genetically related.

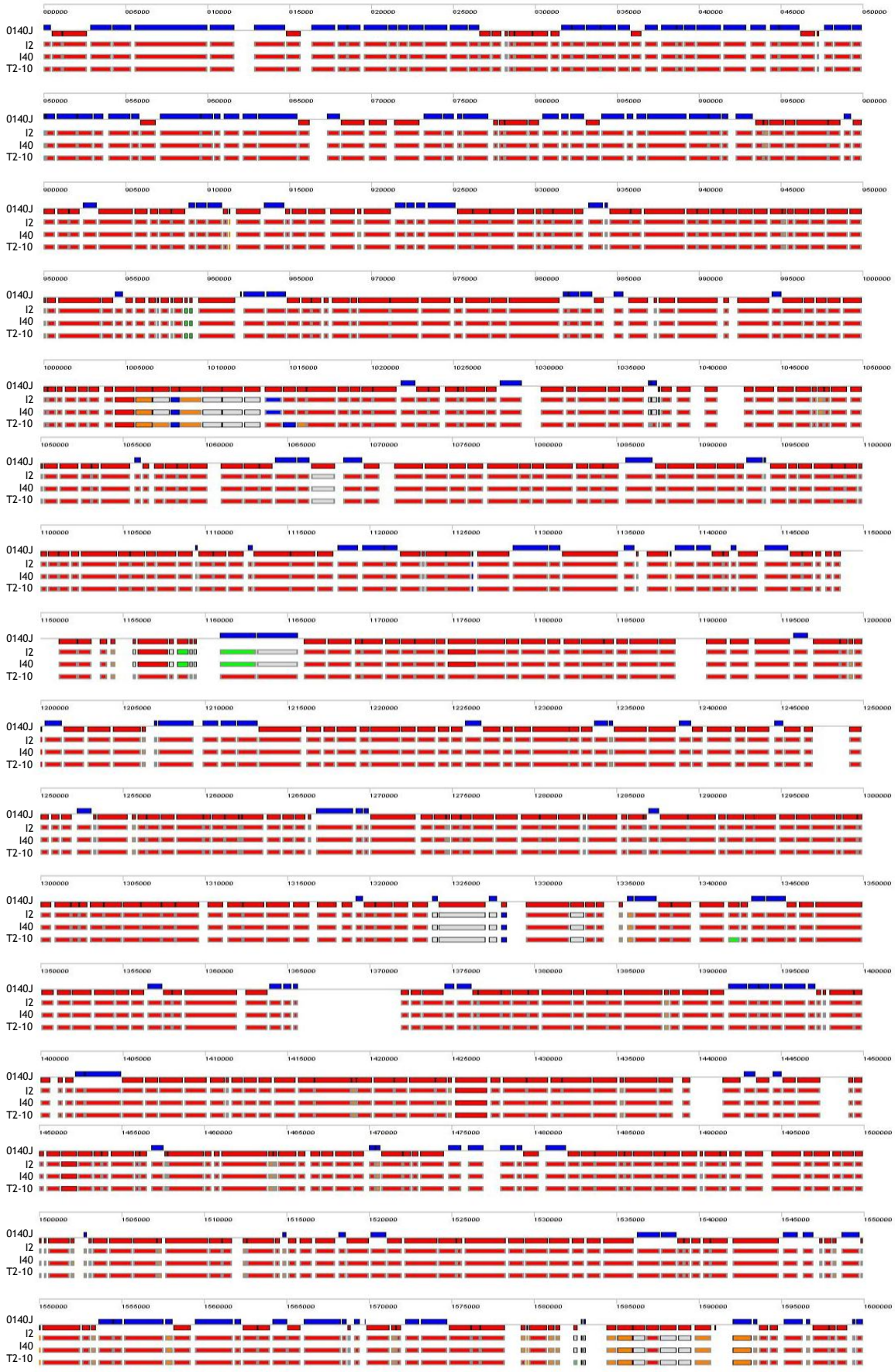
3.2.11 Whole Genome Sequencing

To explore the global differences between *S. uberis* isolates, rather than limiting analysis to specific genes, whole genome sequencing was completed for five diverse isolates. Sequenced isolates included the reference strain 20569, two UK isolates and two Italian isolates. The UK isolates sequenced were: T2-10, from a persistent bovine infection, typed as ST 6 and a member of the dominant UK ST 5 CC and T1-60 (ST 345), from a non-persistent infection and not a member of any CC. The Italian isolates sequenced were: I2 (ST 293) and I40 (ST 305) from ovine and bovine mastitis cases respectively. Isolate I40 was a member of the dominant Italian group (ST 305) whilst I2 was a singleton in all BURST analyses. At the time of writing, only sequence data for three of the isolates, I2, I40 and T2-10, was available for preliminary analyses. Assembled genome sequences were not circular, but rather were considered to be more than 90 % complete, represented by approx. 100 contiguous sequences (contigs) for each genome. Single nucleotide polymorphism (SNP) analysis of the 3 genomes was conducted, as compared to the published *S. uberis* 0140J reference genome sequence. A total of 11,941, 9,250 and 4,814 SNPs were identified for I2, I40 and T2-10 respectively. Perhaps unsurprisingly, the Italian isolates displayed the highest numbers of nucleotide deviations from the *S. uberis* 0140J reference genome, whilst the UK isolate, which belonged to the ST 5 CC (of which 0140J is also considered to be a member) showed the least nucleotide changes. Further details regarding SNPs and information about the processing of these samples can be found at:
http://xyala.cap. ed.ac.uk/Gene_Pool/2009082_Tamara_Lang_eurvcsf8/processed/

The sequence data for the three genomes were assembled by concatenating contigs with more than 999 bps, with the region between each contig being padded with strings of 50 × 'n' to readily denote the distinction. No effort was made to assemble the contigs into any meaningful order, since it was unclear how similar the genomes were to the only other sequenced *S. uberis* genome, that of 0140J. Instead, a software package developed in-house at Moredun was utilised to allow a comparison of query DNA sequences against a reference sequence. Initially, open-reading frames (ORFs) were predicted in each concatenated genome. Subsequently, the three genomes, and that of 0140J, were subjected to comparative analyses using GeneRator (Beta version, Moredun Bioinformatics Unit, Moredun Research Institute, http://bioweb/GeneRator/Generator_welcome.htm). This software package allows the presence or absence of ORFs within a reference sequence to be determined within a query sequence (or sequences), irrespective of where in the query sequence the homologous ORF lies (hence there being no necessity to assemble the new *S. uberis* genomes in a meaningful order). Furthermore, where homologous ORFs are present in query sequences, the overall level of homology is indicated. Since all query genomes are compared to the reference genome, only the ORFs within the reference genome are considered, however, and hence novel sequences within the query genomes are missed. Consequently, for any comparison, it is necessary to conduct multiple analyses in order to identify novel ORFs within each new sequence. In total, 4 distinct analyses were conducted, using each of the novel *S. uberis* sequences, and that of 0140J, as the reference sequence.

Using 0140J as the reference genome highlighted very little by way of difference between it and the other genomes, in that the majority of coding sequences present in 0140J were also present in the other genomes. In **Figure 3.15**, the comparison of the three novel genomes against 0140J is presented as an example of the GeneRator output. Conserved ORFs with a high level of homology to the reference sequence are highlighted in red, with reducing homology indicated by different colours, down to white boxes to indicate absence of an ORF. Equivalent analyses were conducted using each of the genomes as the reference sequence. There was not sufficient time remaining within this project to undertake a full and thorough analysis of the differences between each genome. Rather, the purpose was primarily to determine the extent of genomic rearrangement between different *S. uberis* strains, and to identify gross differences between each genome. GeneRator analysis, conducted using the novel genome sequences as the reference genome identified more in the way of non-conserved regions, as exemplified in **Figure 3.16**. As a preliminary analysis, a list of ORFs found within the new genome sequences, but not 0140J, was compiled. The translated


0140J
I2
I40
T2-10


0140J
I2
I40
T2-10

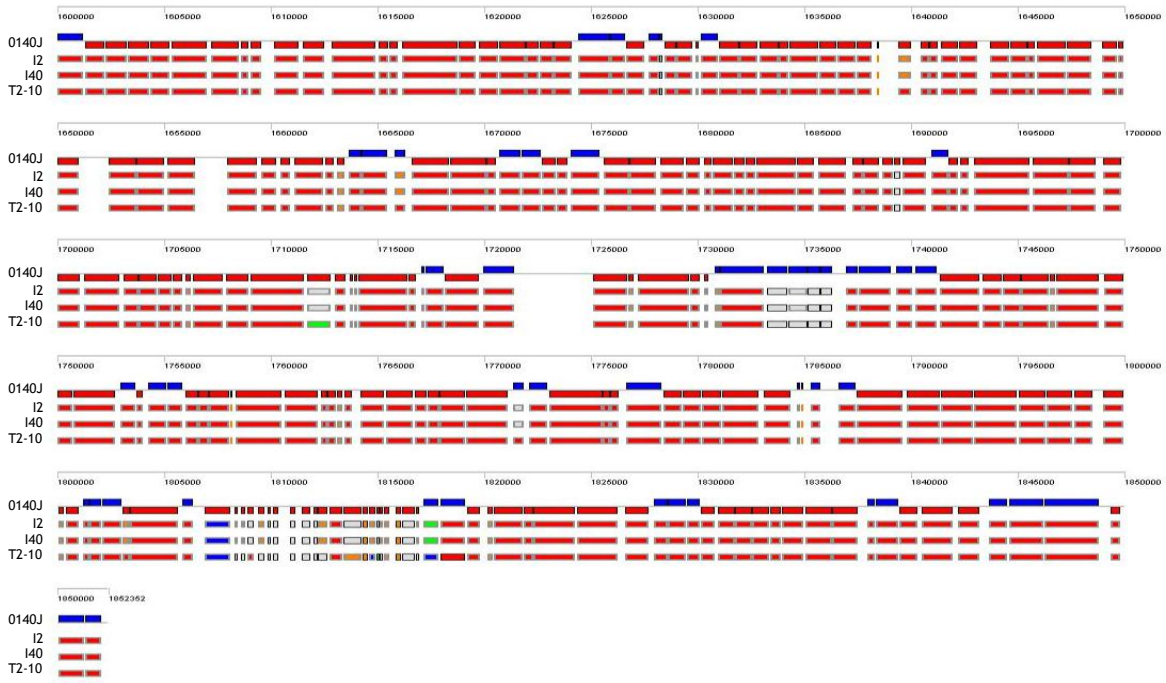


Figure 3.15: Comparison of *S. uberis* 0140J genome to sequence data from genomes of *S. uberis* isolates I2, I40 and T2-10 using GeneRator. The top bar represents the complete genome of *S. uberis* 0140J, with the blue bars corresponding to ORFs on the forward strand and the red bars corresponding to ORFs on the reverse strand. The next 3 rows represent the sequence data from isolates I2, I40 and T2-10, with the colour of the bar providing an illustration of the homology between the DNA sequence from the isolate and the reference strain (0140J). Whilst the ORFs from 0140J are in order, the sequence data from the remaining strains are not necessarily in the corresponding order. It is clear from this illustration that all three isolates share high homology with *S. uberis* 0140J at most regions of the 0140J genome. Where it appears that an 0140J gene region is absent from one or more of the mastitis isolates, this may simply be because the gaps in these genomes have yet to be closed, so these regions might still be present.

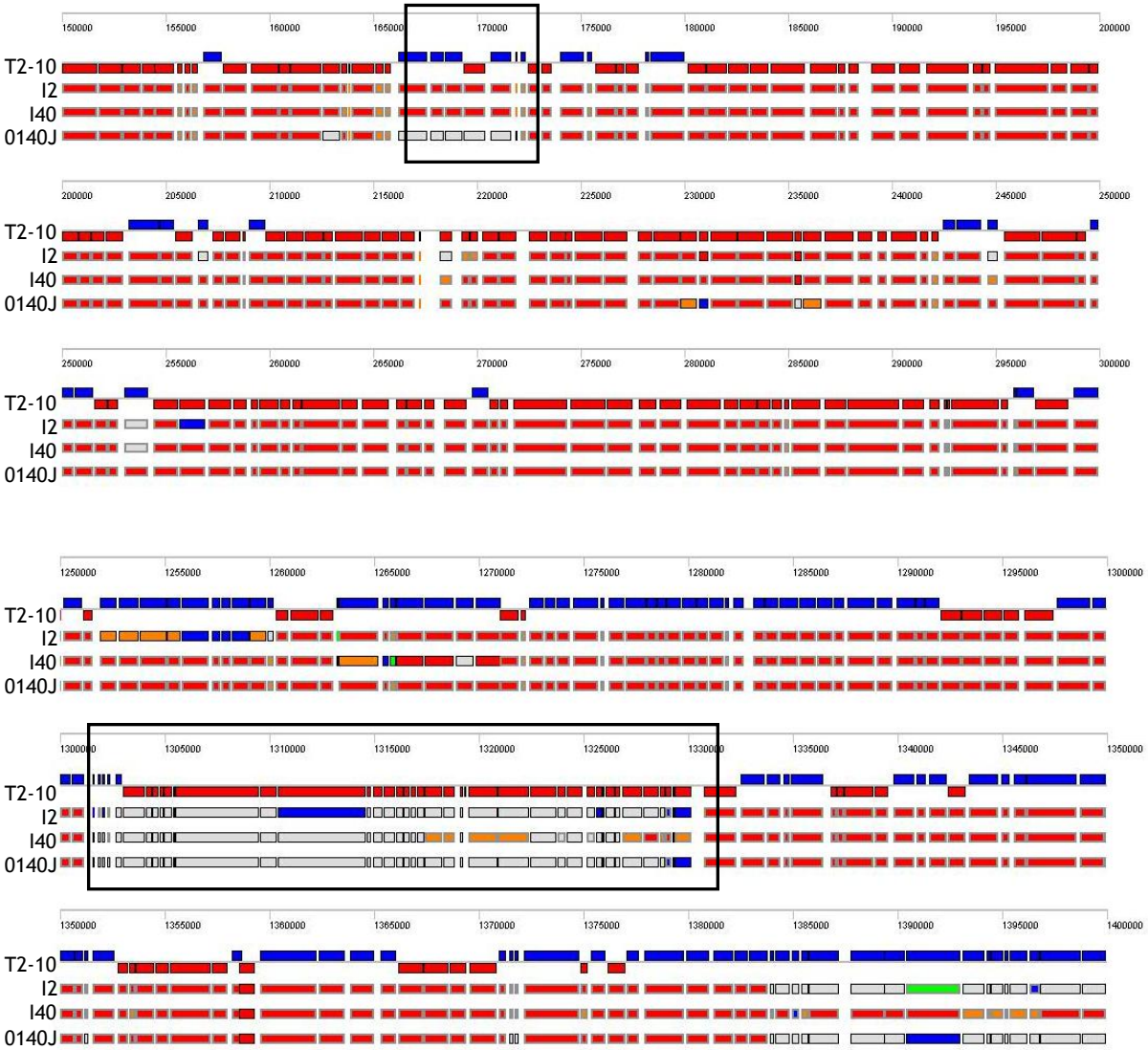


Figure 3.16: Regions from GeneRator comparison of *S. uberis* isolates I2 and I40 and strain 0140J to isolate T2-10. The colour of the bars for the first isolate (T2-10), are blue or red dependent upon which strand the ORF lies on. The colour of the bars for the remaining isolates is an illustration of the homology shared by the isolates at this region compared to the reference isolate, in this case T2-10. A region where several putative genes are present in all isolates except 0140J is identified within the top box. The amino acid sequence data for these genes was selected in GeneRator and subjected to a BLAST search; the translated product of one of these genes shared the greatest homology with a putative Mg^{2+}/citrate transporter protein from *S. mutans*. The second boxed region identifies a large group of genes which appear to be present only in T2-10. These genes may yet be present in the other newly sequenced isolates upon completion of the genome sequences, but they are definitely not present in 0140J. Amongst these highlighted genes were translated products with high homology to several streptococcal phage proteins, including putative phage-associated cell wall hydrolase and phage terminase (small and large subunit) proteins.

products of these ORFs were then identified by BLAST interrogation of the NCBI non-redundant database, and are presented in **Table 3.10**. Interestingly, most of the identified genes encoded proteins not immediately identifiable as classical virulence factors. Rather, they were largely associated with nutrient acquisition. A large proportion of the novel genes (87 %) were most-closely homologous to those already described in other streptococcal species. Several regions were also identified that were apparently unique to a single isolate (although this cannot be guaranteed as the gaps in the new genome sequences have not been closed at this time). In these regions, phage-derived sequences were typically discovered.

Table 3.10: Reciprocal best hit matches for 'genes' identified in all three sequenced *S. uberis* genomes which were not found in the completed *S. uberis* 0140J genome.

Reference	Gene Product	Organism
NP-688885.1	Sugar-binding transcriptional regulator RegR	*S. agalactiae* 2603V/R
NP-688887.1	PTS system, IID component	*S. agalactiae* 2603V/R
NP-688888.1	PTS system, IIC component	*S. agalactiae* 2603V/R
NP-688889.1	PTS system, IIB component	*S. agalactiae* 2603V/R
NP-688891.1	PTS system, IIA component	*S. agalactiae* 2603V/R
NP-721408.1	Putative Mg^{2+}/citrate transporter	*S. mutans* UA159
NP-735119.1	Sugar transporter, putative	*S. agalactiae* NEM316
YP-001620901.1	Chromate transport protein	*Acholeplasma laidlawii* PG-8A
YP-002285934.1	Citrate lyase beta chain	*S. pyogenes* NZ131
YP-002744407.1	Surface-anchored 5'-nucleotidase	*S. equi subsp. Zooepidemicus*
YP-002744873.1	Penicillin-binding protein 2b	*S. equi subsp. Zooepidemicus*
YP-002745282.1	Group II intron reverse transcriptase/ maturase	*S. equi subsp. Zooepidemicus*
YP-002746506.1	GntR family regulatory protein	*S. equi subsp. equi* 4047
YP-002996325.1	Ammonium transporter (Amt) family protein	*S. dysgalactiae subsp. equisimilis* GGS_124
YP-002996708.1	Putative ammonia monooxygenase	*S. dysgalactiae subsp. equisimilis* GGS_124
YP-003429802.1	Putative glycosyltransferase	*S. gallolyticus* UCN34
YP-139367.1	Type III restriction-modification system methylation subunit	*S. thermophilus* LMG 18311
ZP-00782418.1	Reticulocyte binding protein	*S. agalactiae* H36B
ZP-00785181.1	Glucuronyl hydrolase	*S. agalactiae* COH1
ZP-00787477.1	Reticulocyte binding protein	*S. agalactiae* CJB111
ZP-00789090.1	Transcriptional regulator, Cro/CI family	*S. agalactiae* 515
ZP-04009888.1	Abortive phage resistance protein	*Lactobacillus salivarius* ATCC 11741
ZP-05747533.1	YeeE/YedE family integral membrane protein	*Erysipelothrix rhusiopathiae* ATCC 19414

3.3 Discussion

Multi-locus sequencing typing is a widely used epidemiological tool for exploring the relationships between strains of the same species. This can be particularly useful for monitoring disease outbreaks (Camargo *et al.*, 2006; Ruiz-Garbajosa *et al.*, 2006), exploring changes in populations over time (Jefferies *et al.*, 2010), distinguishing between clinical and environmental or pathogenic and non-pathogenic strains (King *et al.*, 2002; Kotetishvili *et al.*, 2002), and data can even be correlated to the carriage of antibiotic resistance genes to monitor their spread (Cookson *et al.*, 2007). Several MLST studies have analysed isolates of *S. uberis* from cases of bovine mastitis and the cow environment (Coffey *et al.*, 2006; Lopez-Benavides *et al.*, 2007; Pullinger *et al.*, 2007; Pullinger *et al.*, 2006; Rato *et al.*, 2008; Tomita *et al.*, 2008; Zadoks *et al.*, 2005a). Despite the consistent isolation of multiple STs, in both the UK (ST 5 CC) and New Zealand (ST 143 CC) dominant CCs have been identified comprising several frequently isolated STs (Coffey *et al.*, 2006; Pullinger *et al.*, 2006) and in Australia these CCs were positively associated with isolates from clinical and sub-clinical mastitis (Tomita *et al.*, 2008).

Supplementary observations made during this study included the identification of 4 isolates, three of which were from Italy, in which the housekeeping *yqiL* allele was absent. A previous *S. uberis* MLST study similarly identified *yqiL* negative isolates in 4 of 46 Australian isolates, and subsequently identified a homologous gene with similar predicted function, elsewhere in the *S. uberis* 0140J genome (Tomita *et al.*, 2008). A small number of *yqiL* deficient *S. pyogenes* isolates were also found and subsequently observed to rapidly disseminate amongst the population becoming the most prevalent clone identified (Perez-Trallero *et al.*, 2007). These observations suggest that the *yqiL* gene product is not in fact essential for survival, and that the loss of this gene may offer some benefit, perhaps by reducing the organism's metabolic load. Amplification and sequencing of the alternative acetyl-coA acetyltransferase gene from isolates in this study from which *yqiL* was both present and absent, confirmed that *yqiL* is not vital to the survival of the bacterium, as an alternative option for the generation of this enzyme is present. Alternatively, the loss of *yqiL* may instead be associated with the gain of additional genes which provide benefits for survival, such that loss of *yqiL* is not in itself directly related to 'fitness' but happens to be located in the region in which genetic exchange occurred. Increased absence of *yqiL* carriage would in this case simply correspond to increased spread of advantageous genes which replaced *yqiL*. For example, it seemed that for *S. pyogenes*, the loss of *yqiL* corresponded with the gain of

alternative resistance genes, and thus, this clone was subsequently rapidly disseminated (Perez-Trallero *et al.*, 2007). Whole genome microarrays have recently identified core and variable regions of the *S. uberis* genome and the *yqiL* gene was in fact located within one (RD20) of these regions (Lang *et al.*, 2009). As the variable regions are likely to determine virulence and encode cell surface proteins, it is thus not unexpected that *yqiL* could be lost during recombination or horizontal gene transfer at this locus.

A further interesting observation from this study was the finding that 100 % of UK (but just 84 % of Italian) *S. uberis* isolates possessed the virulence gene, *pauA*, which has been investigated for suitability as a mastitis vaccine (Leigh *et al.*, 1999). A single Danish *S. uberis* isolate was previously shown to lack *pauA*, and in this isolate a novel, broader-spectrum plasminogen activator, termed *pauB*, was identified (Ward and Leigh, 2002). Subsequent studies have failed to amplify a *pauA* product in 4 % (Zadoks *et al.*, 2005a) and 3 % (Khan *et al.*, 2003) of mastitis isolates, respectively. It seemed likely that all these isolates may also possess *pauB*, and indeed all Italian isolates from this study did, but, the *pauA*-negative isolates from the Zadoks *et al*. (2005a) study were all negative for *pauB* (data not shown). Of the *pauB*-positive isolates identified in this study, the majority were isolated from ovine mastitis cases, while a few originated from cases of bovine mastitis. Interestingly, it has been shown that PauB has an enhanced capacity to activate ovine plasminogen over its PauA counterpart (Ward and Leigh, 2002), and acquisition of *pauB* may therefore confer a competitive advantage to pathogenic strains causing ovine mastitis. In contrast, both PauA and PauB have equivalent capacities to activate bovine plasminogen, and hence carriage of *pauA* or *pauB* by bovine-pathogenic strains would make no difference to bacterial 'fitness'. In this study, all *S. uberis* MLST isolates tested carried either *pauA* or *pauB*. While the results presented here offer tantalising evidence that the plasminogen activator (either A or B) may contribute to virulence, certain *pauA* negative isolates (Zadoks *et al.*, 2005a) were shown not to harbour *pauB* (that is not to say that they do not utilise an alternative, as yet un-described plasminogen activator), and previous work has shown that PauA is not required for the survival of *S. uberis* in bovine milk or the bovine mammary gland (Ward *et al.*, 2003). No equivalent study has, however, been conducted to determine the impact of *pauA* or *pauB* deletion on bacterial survival in the ovine mammary gland.

It has previously been suggested that the primary mechanism of evolution within *S. uberis* is via horizontal gene transfer, and the acquisition of *pauA* is believed to have arisen as a result of prophage integration (Ward *et al.*, 2009; Zadoks *et al.*, 2005a). This

hypothesis was supported by observations, presented here, that many novel genes unique to the newly sequenced *S. uberis* genomes were found, and that many of these were within regions containing phage-derived sequences. The acquisition of *pauB* may similarly be attributed to horizontal gene transfer, and whilst its carriage may offer no benefit for the infection of cows, explaining its low frequency, it does appear to be beneficial for the infection of sheep, hence it has disseminated amongst an otherwise genetically diverse (as determined by MLST) ovine population. Additional sampling, particularly from ovine populations, and PCR screening would support this hypothesis. Evidence for transfer between different sub-populations demonstrates the importance of typing strains from diverse hosts, as this represents an additional reservoir for genetic exchange, which may impact upon vaccine efficacy.

In the data presented here, typing of UK isolates revealed that ST 5, 6 and 20 were predominant, accounting for 11, 7 and 5.5 % of the collection respectively. These strains have also been isolated previously from multiple cows on multiple farms around the UK (and once from Sweden), and represent 29 % of the UK isolates characterised on the MLST database. Most interestingly, these STs were found in the same herd at two distinct sampling times and from both short and prolonged untreated infections (Coffey *et al.*, 2006; Pullinger *et al.*, 2007). In this study these STs were similarly identified from both antibiotic-cured (non-persistent) and persistent infections unresponsive to antibiotics. Strains exhibiting the most common allele at each locus are extremely unlikely to arise by chance, given the high levels of discrimination achievable by MLST; where isolates differing by just a single nucleotide are assigned to unique STs (Enright and Spratt, 1999; Spratt, 1999). The predominance of these strains thus may denote that they are well suited for survival in the mammary gland allowing them to be widely propagated and thus frequently found associated with mastitis.

Characterisation of STs found on a single UK farm revealed that more than 80 % of isolates shared high genetic similarity, with STs 5, 6 and 20 unsurprisingly predominant. The large number of variants sharing 6 of 7 alleles, found on the same farm, suggests recent diversification of novel but genetically similar types from the same predominant founding genotype (ST 5) which has presumably arisen due to increased fitness (Feil *et al.*, 2004; Spratt *et al.*, 2004). Persistence on this farm, of a strain in one animal, may also promote infection of other animals, as, the dates during which persistent animals were infected, in some cases, coincided with the dates of non-persistent infections with the same ST in other animals. Previous research has also suggested that contagious

transmission can contribute to the spread of *S. uberis* on a single farm (Zadoks *et al.*, 2001).

The persistent UK isolates used in this study had previously been characterised using Restriction Endonuclease Fingerprinting (REF), and the same or similar profiles were found in replicate isolates from 96 % of cases, although the profiles from distinct infections were also similar (Milne *et al.*, 2005). The same multi-locus ST was, however, assigned to all sequential isolates from 68 % of infections which persisted despite antibiotic therapy and this was, for the most part, confirmed by RAPD typing. These results suggest that the discriminatory power of REF was insufficient to differentiate between closely related strains. In another study, it was found that 70 % of extended, untreated infections were caused by the same *S. uberis* ST (Pullinger *et al.*, 2007), a remarkably similar value to that reported here from persistent infections not cured following antibiotic therapy. This observation suggests that antibiotic therapy may have little effect on *S. uberis* once it has established itself within the mammary gland, with cases just as likely to be eventually resolved naturally by the host. Clearly, to support this claim, a negative controlled treatment trial would, however, be required. This observation also clearly demonstrates that *S. uberis* has adapted to evade host factors and antibiotics. No specific STs were responsible, however, for persistent infections; and indeed greater diversity was found within this population over the non-persistent population. Multiple methods for resisting host defences or antibiotic therapy, permitting long-term survival in the mammary gland may thus be utilised by *S. uberis* explaining the highly divergent genotypes identified. Alternatively, a single factor may be responsible for persistence, this being acquired and horizontally transferred between strains, allowing persistence in a wide collection of genetically diverse strains. Acquisition of a single 'virulence' factor could also explain the observation, made in this study, that *S. uberis* isolates sharing the same ST were derived from both persistent and non-persistent infections and that these isolates did in fact differ in their RAPD profiles.

Whilst no obvious trend between ST and persistent infection was found, several small groups, identified by BURST were associated with only non-persistent infections. No groups were associated with persistent infections only; however, 65 % of persistent isolates belonged to the ST 5 CC, although 54 % of non-persistent isolates were also associated with this CC. The predominance of the ST 5 CC cannot thus be simply explained by the ability of these strains to resist antibiotics *in vivo* as not all strains were from persistent infections. By comparison, dominant *S. aureus* mastitis genotypes were not associated with a specific *in vitro* antimicrobial profile (Mork *et al.*, 2005) and

indeed more that 99 % of the isolates used in the work presented here, have been shown previously to be sensitive to penicillin G and cefquinome *in vitro* (Milne *et al.*, 2005). Ribotyping of Finnish *S. uberis* isolates found *in vitro* resistance to erythromycin and oxytetracycline was predominantly limited to two ribotypes (Pitkala *et al.*, 2008). A small fraction of UK *S. uberis* isolates previously typed by MLST were assigned to the ST 86 CC which predominantly comprises New Zealand isolates (Pullinger *et al.*, 2006). In the data presented here, a small fraction of UK mastitis isolates (5 %) were also assigned to the ST 86 CC, and all these isolates were obtained from non-persistent infections. Interestingly, in a collection of Australian isolates, the ST 5 CC was found to be highly associated with virulence, whilst ST 86 CC was found not to be associated with cows with clinical or sub-clinical mastitis, but with cows with low cell counts (Tomita *et al.*, 2008). Similarly, the ST 143 CC was associated with high virulence (Tomita *et al.*, 2008) and in the data presented here, a single ST (representing 3 isolates from the same persistent infection) was assigned to this CC. Typing therefore appeared to offer some tentative correlation with phenotype, although this was by no means definitive. As this was not the intended function of this technique, it is perhaps not surprising that MLST alone cannot track virulent, persistent strains or *in vivo* antibiotic resistance. In cases where MLST groupings link to clinical features or specific phenotypes, this is most likely to be the result of indirect linkage of the MLST targets with the relevant genes (Turner and Feil, 2007). Virulence gene carriage can instead be subsequently correlated with STs or CCs where they may prove to be relevant. Observations that the *hasA* capsule gene predominates in the dominant UK and New Zealand CCs demonstrates this point (Coffey *et al.*, 2006; Pullinger *et al.*, 2006). Similarly, strong correlations between virulence determinants or antibiotic susceptibility with RAPD types of *S. aureus* mastitis isolates have been observed (Fitzgerald *et al.*, 2000).

The MLST database groups isolates into a CC if they share at least 4 alleles with the group founder (Coffey *et al.*, 2006), whilst a biologically meaningful CC, representing recent diversification of a group of STs from a common founder, is considered to be a BURST group where isolates share 6 of 7 alleles (Feil and Enright, 2004). Analysis of the UK collection identified a large main group of related isolates which was highly similar when either of these CC definitions was used, as well as when the group definition was set to isolates sharing 5 of 7 alleles. A previous MLST study from the UK identified that the ST 5 CC comprised 70 % of the UK population studied (Coffey *et al.*, 2006) and in the data presented here, 54 % of UK isolates were assigned to the ST 5 CC including most of the STs identified more than once. An exception was obvious in ST 20 which shares just 2 alleles with the founder of the ST 5 CC. Setting the group definition as isolates sharing

5 of 7 alleles identified additional, smaller, closely related groups within the UK collection, and ST 20 was a member of one of these groups. A population snapshot also suggested that this frequently isolated ST was diversifying, perhaps implying the origins of a new CC.

Within the Italian population, no STs were shared between isolates from ovine and bovine hosts. This is unlike *S. aureus*, where the most common genotypes have been shown to exhibit no host preference (Mork *et al.*, 2005). Subsequent BURST analysis did show, however, that some Italian STs were closely related to UK bovine STs. In the Italian ovine population, there was no ST that was represented by more than one isolate, except where isolates were from the same farm. Although the ovine Italian population sampled is too small to draw any definitive conclusions, it appears that different strains were responsible for infection on each farm, with the possibility of contagious transmission occurring within farms. This would be consistent with evidence of contagious transmission amongst *S. agalactiae* mastitis isolates (Baseggio *et al.*, 1997; Merl *et al.*, 2003; Wang *et al.*, 1999), and Portuguese *S. uberis* isolates (Rato *et al.*, 2008), and is perhaps due to different hygiene practices and quality regulations for sheep in comparison to cattle (Klinger and Rosenthal, 1997). High genetic diversity amongst ovine isolates predominantly explains the greater diversity amongst Italian isolates in general in comparison to UK isolates. Within the Italian bovine collection of 35 isolates, seven STs were represented by more than one isolate. In three cases STs represented multiple samples from the same farm. Most frequently identified was ST 305 which represented 5 isolates from 3 different farms in the region. In this region of Italy, it thus appears that, as in the UK, a group of strains pre-dominate, frequently being associated with bovine mastitis. Additional Italian isolates from cattle were subsequently added to the MLST database by another group, and interestingly, some STs were shared with those found in this study and some were genetically related to isolates from the dominant CC described here.

No STs were represented by isolates from both the UK and Italian populations analysed in this study, however, a single bovine Italian isolate did share the same ST as a bovine isolate originating from the UK that was previously entered onto the PubMLST database. This is in keeping with results from previous studies where UK, New Zealand and Portuguese isolates exhibited distinct STs (Pullinger *et al.*, 2006; Rato *et al.*, 2008) whilst just 2 STs from Australian isolates were found previously in either the UK or New Zealand (Tomita *et al.*, 2008). *S. uberis* populations thus appear to be highly heterogeneous based on ST alone, with each country exhibiting a unique pool of strains.

Development of a vaccine based upon data from any single country could thus have greatly reduced efficacy in another country (unless it is found that isolates distinct by MLST analysis carry equivalent virulence factors). Of the Italian isolates characterised, only 8 % belonged to the ST 5 CC, however, these isolates were members of the major Italian BURST group identified. A major group was also identified by BURST analysis of both the UK and Italian isolates combined, and in this group, the dominant STs derived from cows in both countries were present. It was thus revealed that, in each country, a sub-set of related STs have become particularly well-adapted to the mammary gland niche and thus are more likely to cause infection (either persistent or non-persistent) within and between herds. A population snapshot of all database STs further demonstrated that dominant CCs from the UK and New Zealand were also related forming a single, central complex. This is surprisingly similar to typing analysis of bovine *S. aureus* mastitis isolates, where a single CC and a few specialised clones from distinct countries were dominant (Fitzgerald *et al.*, 1997; Smith *et al.*, 2005), suggesting that these related strains may in fact have increased virulence.

The number of novel alleles identified at each locus was higher in the Italian collection, but as the MLST database contains a large number of UK isolates this is not surprising. It does demonstrate, however, that some alleles are country-specific, implying that genetic differences exist between strains from different countries. It can also be seen that the number of alleles identified at each locus is quite similar in both populations, despite the Italian collection being considerably smaller (potential hypotheses for this observation are discussed later). The mean number of alleles per locus was 16, implying a potential to differentiate up to 2.7×10^8 different STs. This was higher than in the first MLST study using the standardised protocol where an average of 10 alleles per locus was found (Coffey *et al.*, 2006). The nucleotide diversity at some "housekeeping genes" such as *tdk* was higher than expected and showed even greater heterogeneity than that seen at the *gapC* locus. The multi-functions of GapC have been well described (Madureira *et al.*, 2007; Terao *et al.*, 2006) and its extracellular location and virulence role explain the diversity observed here, as the extracellular protein will be subject to greater change; yet with GapC essential for cell survival (Gase *et al.*, 1996; Winram and Lottenberg, 1998), changes affecting the enzyme function will not be maintained, hence a relatively low dN/dS ratio is also observed. Despite high heterogeneity, a low dN/dS ratio was identified at the *tdk* locus, implying that this gene is not subjected to a high selection pressure; the predicted number of recombination events at this locus was, however, high. Clearly, the degree of heterogeneity attributable to recombination and mutation varies widely at the different gene loci analysed. Evidence for *pauA*

positive selection was observed, in line with previously published data (Zadoks *et al.*, 2005a). Despite low heterogeneity at the *tpi* allele, the number of non-synonymous mutations was high and thus this allele had a surprisingly high dN/dS value, suggesting a greater selection pressure for this gene. This observation may be attributable to the hypothesis that *tpi*, like *gapC*, encodes a multifunctional protein which can reside in an alternative location and carry out an alternative role, thus may be under greater pressure to frequently adapt to combat host defences, or improve its function. Unlike GapC though, some changes in the Tpi amino acid sequence, clearly do not detrimentally affect the glycolytic function of the protein. The function of many so called 'housekeeping' gene products may thus need to be reconsidered, taking into account any additional virulence functions the proteins may have. A review by Pancholi and Chhatwal (2003) further discusses the precedent for housekeeping enzymes to exhibit multiple functions and considers that new strategies may be utilised to block the translocation of these enzymes to the pathogen surface which may have the potential to control infection.

Bacterial diversification is largely mediated by the following processes: homologous recombination, in which DNA regions flanked by sequences homologous between donor and recipient can be exchanged; illegitimate recombination between short sequences of little or no homology resulting in DNA rearrangement that is enhanced by DNA damaging events; or lateral gene transfer, where novel DNA is introduced from a completely unrelated source (also termed horizontal gene transfer). Mutation events produce further variation amongst strains. By definition, mutation or gene changes arising from homologous recombination may be detrimental, beneficial or neutral in effect. If a mutation is detrimental, the strain becomes less fit, and hence will be gradually lost from the originating population. If the genetic change is neutral in effect, then no benefit is granted to the altered strain, and it will be found within the originating population at an equivalent rate. In contrast, if the genetic change offers a benefit to the organism, by, for example, improving function of an existing gene, then the new allele will subsequently be found in high numbers within the population. Horizontal gene transfer, however, facilitates the introduction of a completely new gene, or set of genes, which may offer the recipient an increased ability to survive within its original environment or the ability to exploit an entirely new environmental niche.

Using MLST data alone to reconstruct the evolutionary history of mastitis isolates, in order to determine the emergence of pathogenic clones, is extremely complicated by the clonal divergence caused by both recombination and mutation. In this study, linkage

disequilibrium was detected, implying a clonal population; however, recombination was also evident from the lack of congruence between gene trees; thus the *S. uberis* population may be said to be weakly clonal. A high frequency of homologous recombination at one gene region, to increase the spread of an advantageous gene does, however, remain consistent with observations of linkage disequilibrium elsewhere on the genome (Lawrence, 2002). It has also been demonstrated that linkage disequilibrium is found erroneously in highly recombining populations due to the presence of many closely related genotypes in the overall sample set (Ruiz-Garbajosa *et al.*, 2006). The manner in which diversification occurs is species dependent, but it was found that recombination was significantly more likely to produce variant *S. pneumoniae* and *Neisseria meningitidis* alleles and nucleotides than mutation (Feil *et al.*, 2000).

The ability of BURST to correctly identify the evolutionary descent of isolates within a population (and thus place them into closely related groups) is also vastly affected by the proportion of allelic variation which arises as a result of point mutation compared to recombination (Turner *et al.*, 2007). No BURST groupings can be identified for highly recombining populations with a high rate of mutation, such as *Helicobacter pylori* (Suerbaum *et al.*, 1998). Alternatively, highly recombining species with a lower rate of mutation can be grouped, but potentially incorrectly, into a single, large, 'straggly' group (Turner *et al.*, 2007). Population snapshots of *S. uberis* database STs identify such a single 'straggly' group, suggesting that BURST may be grouping STs which share several alleles due to recombination rather than common ancestry. The apparent increased diversity amongst the Italian population, over that from the UK, observed in the work presented here, may merely be a manifestation of this limitation of BURST. Due to the larger UK group size, BURST is able to identify a large closely related main group of isolates with similar allelic profiles; but this group may in fact represent a false impression of relatedness, as these strains may only appear homologous due to high levels of recombination. Clearly, in the Italian population an equivalent number of alleles have been identified at each locus, implying that this population has greater diversity. This population is however much smaller and from bovine and ovine mastitis cases, so, if additional sampling was completed it may simply be the case that only a limited number of additional alleles would be identified. Few groups could be observed in the Italian collection, again because of high levels of recombination, but the allelic profiles which may determine the links (albeit potentially inaccurate ones) between STs have yet to be identified. The observation of additional Italian isolates (from unrelated studies) sharing homology with isolates from this study, supports this hypothesis.

Alternatively, it may simply be found that the Italian population is diversifying at a greater rate than the UK population, either as a result of a higher mutation rate, or a higher rate of recombination.

MLST is accepted to be an unambiguous and reproducible protocol for the characterisation of bacterial populations. The process is also claimed to be inexpensive, but in this study, sequencing costs were extensive (although it is acknowledged that cheaper options may be available). Data analysis is laborious, and programmes such as START and DnaSP are not particularly intuitive. Results obtained by MLST (as with any typing system) are also highly dependent upon the collection sampled and the epidemiological source data available for these isolates. A large local collection of isolates are, for example, more likely to contain fewer STs than a smaller collection of individual isolates from diverse regions. This must be considered when inferring relationships between isolates. Furthermore, isolates sharing the same ST are not necessarily homologous. Identical STs have frequently been shown to exhibit different PFGE types (Lang *et al.*, 2009; Rato *et al.*, 2008; Tomita *et al.*, 2008) and comparative genome microarray has demonstrated that strains of *S. pneumoniae* with identical STs are genetically distinct and exhibit variable virulence in mice (Silva *et al.*, 2006). Isolates sharing the same ST are thus merely homologous at defined gene regions, which in the case of housekeeping genes normally lie within the core genome (*yqiL* being an obvious exception). As this is not representative of the entire genome, in MLST, unlike PFGE, isolates of the same ST may be identical in the core genome, but may be grossly different in the remaining variable regions; it is precisely these variable regions which are likely to define pathogenicity. The typeability of the isolates included in this study was 97.7 % owing to the presence of *yqiL*-negative isolates. Whilst in the *S. uberis* PubMLST scheme, isolates which lack a target gene are currently not assigned a ST, some MLST schemes do assign STs to these isolates so that they can still be included in subsequent analysis and their dissemination monitored (Perez-Trallero *et al.*, 2007). This should be considered for the *S. uberis* PubMLST scheme also. These observations, as well as the extreme differences in heterogeneity at *recP* and *tdk* loci, highlight the importance of allele selection for MLST schemes. Further research would determine if it was pertinent to replace the *yqiL* target with *thlA* (encoding acetyl-coA acetyltransferase) or *gapC* to increase the typeability of *S. uberis* isolates by MLST, whilst it is clear that *pauA* is not a suitable gene target for MLST (although it may be interesting to monitor the presence or absence of this gene in the *S. uberis* population). As an *S. uberis* genome has now been sequenced, gene targets more widely dispersed, thus providing greater coverage of the genome, might provide more accurate

discrimination of isolates and thus represent better MLST targets; currently the standardised scheme only considers around half of the genome (**Figure 3.1**). Greater coverage of one genome may not necessarily, however, correspond to equivalent coverage of another heterologous genome and of course, they may still differ grossly at other loci. This was in part why genome sequencing of further isolates was conducted towards the end of this study. Unfortunately, however, while the genomes were found to be largely homologous in terms of the total complement (with the exception of horizontally-acquired genetic elements), time was not available to close the gaps between the sequenced contigs, allowing determination of the overall order of genes within each chromosome, which may differ widely between isolates. Whole genome microarray hybridisation approximated that 82.5 % of the *S. uberis* 0140J genome was homologous to gDNA from 21 diverse strains (Lang *et al.*, 2009), although, again this technique could not identify if the gene order was also conserved and if novel genes were present in the test strains which were absent from the reference strain (0140J). Considering that initial analysis of the genomes of *S. uberis* isolates (sequenced as part of this thesis research) identified several genes that were novel and not found in 0140J, this suggests that alternative methods to identify genome differences are required.

Despite the caveats associated with MLST, the high reproducibility and value of a standardised scheme was demonstrated however, as the allelic profile of the *S. uberis* reference strain 0140J obtained during this study was identical to that found on the PubMLST database for this strain submitted by another group. Research regarding *S. uberis* has frequently been based upon observations of strain 0140J, the genome of which was sequenced and published in 2009 (Ward *et al.*, 2009). Recent MLST analyses have demonstrated, however, that this strain is not frequently associated with mastitis or the dairy environment, in the UK or in any other country, as from the 857 database isolates (at the time of writing) ST 1 (0140J) was not represented by a single additional isolate. Furthermore, population snapshots demonstrate that this ST was distinct from the more dominant STs of the central complex. This observation may also explain why, within the 0140J genome, well described streptococcal virulence determinants were scarce (Ward *et al.*, 2009). Demonstration of multiple variable regions within the *S. uberis* genome (Lang *et al.*, 2009), evidence of high rates of recombination, and observations made in this and previous studies regarding absent genes such as *yqiL* and *pauA* and the wide diversity within certain individual alleles (Tomita *et al.*, 2008; Zadoks *et al.*, 2005a) demonstrate the need for whole genome sequencing of additional isolates to determine the exact genetic differences between phenotypically distinct isolates. With advances in sequencing technology reducing costs, the ability to sequence

multiple genomes becomes increasingly economically viable and should be considered as the next step for direct comparison of strains; equivalent conclusions have been drawn in several reviews, *e.g.* (van Belkum *et al.*, 2001; van Belkum *et al.*, 2007; Zhang and Zhang, 2006). Despite offering an incomparable capacity to elucidate genetic relationships between strains, a severely limiting factor of genome analyses are the subsequent bioinformatics analyses required to make meaning of the sequence data. It is for this reason that it was not possible within the present study to undertake more in-depth comparisons of the newly sequenced *S. uberis* genomes. The availability of these genomes, however, will facilitate downstream analyses, which may shed further light on the matter of the persistence of some *S. uberis* strains within the udder environment. Furthermore, these genomes may be exploited as part of a "reverse vaccinology" approach, in order to identify antigens which might be suitable vaccine candidates (Capecchi *et al.*, 2004; Rappuoli, 2000), as has been successful for *Neisseria meningitidis* (Giuliani *et al.*, 2006).

The diversity of *S. uberis* types identified in this study was vast, with 99 STs identified from 176 isolates from the UK and Italy, with many being unique to a single isolate. These observations support the growing hypothesis that *S. uberis* is commensal in the bovine environment. The high frequency of recombination identified by MLST is, for, example believed to be in keeping with observations from commensal streptococci (Zadoks *et al.*, 2005a) and the lack of 'classical virulence' factors but the myriad of genes with metabolic functions present in the genome suggest a more opportunistic nature to the bacteria (Ward *et al.*, 2009). Frequent isolation of *S. uberis* from multiple cow sites without apparent disease (Cullen, 1966; McDougall, 2005) further supports this theory. Nevertheless, whilst a large pool of *S. uberis* isolates are found to be capable of causing mastitis, it has also been shown that a significant sub-set are genetically related (either by common ancestry or recombination), and certainly specific STs are more frequently identified in each country, suggesting they are better adapted to survival in the mammary gland, be it persistently or otherwise and hence the *S. uberis* population can be described as weakly clonal. These strains must thus possess certain traits that attribute them with an enhanced ability to infect and survive in the mammary gland (an environment which is not the natural habitat of the bacteria). Results from MLST studies of *S. uberis* and *Enterococcus faecalis* (a commensal of the GI tract) are comparatively similar; as despite high heterogeneity, hospital-acquired disease outbreaks were linked to specific *E. faecalis* CCs, but otherwise there was limited correlation between ST and strain source (Ruiz-Garbajosa, 2006). Clearly more research is required to definitively determine differences between phenotypically distinct, apparently genetically similar

isolates, in order to understand the exact route of *S. uberis* transmission in the dairy environment. As the reasons for *S. uberis* persistence could not be determined by MLST (at least using the MLST panel employed in this study), the research presented in the following chapters was conducted to attempt to identify factors contributing to persistence and virulence. In addition, the suitability of an alternative typing method, to either replace or augment MLST is described.

Chapter 4: Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry analysis (BioTyping) of *S. uberis*

4.1 Introduction

Techniques for the identification and speciation of bacteria are numerous and have a wide range of applications, including the monitoring of disease outbreaks (Camargo *et al.*, 2006; Edwards-Jones *et al.*, 2000) and identification of virulent strains (Kotetishvili *et al.*, 2002; Walker *et al.*, 2002), with results impacting upon subsequent vaccination and control strategies. Due to the development of genomic methods, biochemical and serological typing methods have mostly been superseded (Olive and Bean, 1999). Most genomic techniques for speciation remain, however, unsuitable for high-throughput analysis. The current method of choice, MLST (discussed in **Chapter 3**), has simple principles (Maiden *et al.*, 1998), but remains time consuming and expensive, due to the extensive requirement for DNA sequencing. Furthermore, using traditional MLST schemes, discrimination is based upon sequence variation at just seven genetically stable housekeeping genes, the products of which are unlikely to be in direct contact with the host. Typing of bacteria using this method is thus not likely to reveal any significant correlation with virulence, as has been found by previous MLST of *S. uberis* (Coffey *et al.*, 2006; Pullinger *et al.*, 2007) and during this study. Sequencing of whole genomes is clearly the most unambiguous method for characterising genetic variation in bacteria (Ng and Kirkness, 2010; van Belkum *et al.*, 2001); but, this approach currently remains too expensive to be widely applicable to large numbers of isolates (although it is acknowledged that sequencing costs are decreasing rapidly). The development of an alternative, cheap, quick and simple, yet robust typing technique would thus be greatly valued.

In the last decade, proteomic methods have been utilised more routinely to compare bacterial pathogens; allowing identification of proteins differentially expressed in different isolates, or under different conditions, which may prove to be relevant to virulence, and thus represent potential vaccine candidates (Alam *et al.*, 2009; Jungblut, 2001). Comparing the total protein content of bacteria, including proteins in direct contact with the host as well as housekeeping proteins, permits decisions regarding relatedness to be made which are not based purely upon information derived from a small fraction of the cellular content. Differences in protein profiles between *S. uberis* strains may thus explain the variability in pathogenicity or host specificity.

Differentiation between bacteria using MS was demonstrated as early as the 1970s, although at this time significant sample processing was required (Anhalt and Fenselau, 1975). A rapid technique to phenotypically characterise whole bacterial cells using

MALDI-ToF MS was subsequently introduced (Holland *et al.*, 1996; Krishnamurthy and Ross, 1996). This technique, termed intact cell mass spectrometry (ICMS), or intact cell MALDI-ToF MS (ICM-MS), discriminated between different bacteria very quickly and easily, requiring virtually no bacterial processing. Bacterial colonies were spotted directly onto a MALDI target plate, mixed with a matrix solution containing a solvent to solubilise proteins, and ionised using a laser to generate gas-phase ions identified as peaks on a mass/charge (m/z) scale (Claydon *et al.*, 1996; Holland *et al.*, 1996). Peak patterns, essentially 'protein fingerprints', were found to be unique for different bacterial species and even for individual strains of the same species (Krishnamurthy and Ross, 1996; Welham *et al.*, 1998).

Identification of specific ions characteristic of a particular species, termed 'species identifying biomarker ions' (SIBIs) was also demonstrated using ICMS, and were utilised for the identification of unknown bacteria by comparison of SIBIs to reference strains (Holland *et al.*, 1996). Biomarkers for Gram-negative bacteria, different species and even individual strains could be identified (Claydon *et al.*, 1996). Pathogenic species could also be discriminated from related non-pathogenic species (Krishnamurthy and Ross, 1996), demonstrating the power of the MS-based approach. Subsequently, ICMS protocols have been used to identify multiple species from mixed cultures (Madonna *et al.*, 2000) and even to characterise fungal spores (Welham *et al.*, 2000). In most early studies, bacterial cells were prepared directly from agar plates, however, lyophilised cells and cells from liquid culture have also been successfully analysed (Madonna *et al.*, 2000; Wang *et al.*, 1998). Lysis of whole bacterial cells prior to MS equally permitted differentiation of both species and strains (Krishnamurthy *et al.*, 1996).

Proteins account for greater than half of the bacterial cell content (Frantz and McCallum, 1980), thus unsurprisingly, are easily detected using MS, although other cellular components, including small peptides, teichoic acids, oligosaccharides, and lipids may also be ionised. The matrices and acidic conditions used for MALDI MS are also designed to facilitate ionisation of proteins and peptides, generally being found to favour basic, hydrophilic, cytosolic proteins (Fenselau and Demirev, 2001; Ryzhov and Fenselau, 2001). Resulting ions, with molecular masses of greater than 4,000 Da are considered to represent intact proteins. Treating samples, from which distinct mass peaks had been identified by MS, with trypsin, eliminated all peaks from the profile, confirming that peaks are predominantly representative of proteins derived from the cell (Arnold *et al.*, 1999). Identification of individual peaks has been conducted by: sequencing, using computer programmes to match peak molecular masses or tryptic

digests to protein databases, or by comparison to predicted protein masses from genome sequence information (Amiri-Eliasi and Fenselau, 2001; Arnold *et al.*, 1999; Dai *et al.*, 1999; Erhard *et al.*, 1997; Holland *et al.*, 1999). Ribosomal proteins have been found to be particularly abundant on mass spectra of whole cells, with cold shock, acid resistance, DNA binding, metal binding and outer membrane proteins also being identified (Arnold *et al.*, 1999; Holland *et al.*, 1999; Ilina *et al.*, 2010; Pribil and Fenselau, 2005; Ryzhov and Fenselau, 2001; Williamson *et al.*, 2008). In one study, cyclic peptide metabolites from the cell membrane, which are important for virulence, dominated mass profiles from cyanobacteria (Erhard *et al.*, 1997). Generally, however, the identity of individual protein peaks is not determined, although if these have been chosen as SIBIs (Holland *et al.*, 1999) it may be of interest to determine the function of these conserved proteins. Characterisation based upon the distinctive and reproducible fingerprints produced is sufficient to discriminate between species and identify unknown samples, thus the identification of the actual molecules from which these mass peaks derived is largely irrelevant.

Routine re-suspension of cells in acid or acetonitrile matrix mixtures is believed to produce profiles based on cell wall proteins only (Evason *et al.*, 2000; Welham *et al.*, 1998), however, this preparation method has also been shown to disrupt cells of some bacterial species, releasing proteins from inside the cell (Pennanec *et al.*, 2010; Ryzhov and Fenselau, 2001). For characterisation of Gram-positive bacteria, further cellular disruption is deemed especially important as the thick peptidoglycan cell wall appears to mask proteins during ionisation, limiting the number of peaks on the profiles obtained (Smole *et al.*, 2002). Disruption of cells also permits the detection of larger mass proteins (Madonna *et al.*, 2000). Many adaptations of the whole-cell MS protocol have therefore been described, incorporating at least one additional stage during bacterial processing to physically disrupt the cells (Ruelle *et al.*, 2004; Vargha *et al.*, 2006). Lysis using SDS (Wang *et al.*, 1998), ethanol (Madonna *et al.*, 2000), lysozyme (Smole *et al.*, 2002) or further solubilisation with acid, have each been found to enrich the final MS spectra in terms of peaks and mass range. This is presumably due to exposure of intracellular and cell-wall proteins to ionisation. Whilst lysis mostly improved mass profiles obtained from Gram-positive bacteria, the peaks identified from the lysed profile of the Gram-negative species, *Haemophilus ducreyi*, were of lower mass and intensity, with greater background being seen compared to non-lysed cells (Haag *et al.*, 1998). Excessive lysis may thus also cause degradation of exposed proteins; demonstrating the requirement for optimisation to achieve the best results from different organisms. Preparing bacterial cells using different methods will thus result in

positive selection of specific protein populations for ionisation and thus can widely affect the spectral patterns obtained (Vargha *et al.*, 2006; Wang *et al.*, 1998).

Additional factors which may influence protein expression by bacteria and thus may affect MS results are vast, and include the culture medium used (Walker *et al.*, 2002), the stage of bacterial growth from which cells are harvested (Arnold *et al.*, 1999), age of culture/cells (Vargha *et al.*, 2006), salt content (Ruelle *et al.*, 2004), pH (Williams *et al.*, 2003) and extraction solvent utilised (Mandrell *et al.*, 2005; Mazzeo *et al.*, 2006; Ruelle *et al.*, 2004; Wang *et al.*, 1998). The type of matrix, the mass spectrometer and even the operator, might also further influence the final mass spectrum produced (Donohue *et al.*, 2006; Ruelle *et al.*, 2004; Saenz *et al.*, 1999; Wang *et al.*, 1998). When comparing mass data between samples, the importance of an optimised protocol is therefore clear, and ideally all these factors should be kept constant, or at least considered, to allow the most accurate comparison possible. Despite these concerns, the use of different conventional media for the speciation of *Campylobacter* was actually found to have little effect on the SIBIs identified (Mandrell *et al.*, 2005). Similar observations were made regarding mass spectra of *E. coli* (Valentine *et al.*, 2005), *S. aureus* (Bernardo *et al.*, 2002) and *Legionella pneumophila* (Pennanec *et al.*, 2010). Distinctive biomarker peaks were also conserved when using the same protocol over extended periods, when samples were prepared using different methods and even when analysed in different laboratories (Madonna *et al.*, 2000; Saenz *et al.*, 1999; Wang *et al.*, 1998). Contrastingly, peak intensity consistently varies widely, within and between experiments, and as such, limited weight is generally given to the relative intensity of peaks identified, with principally the mass of the derived peaks being considered important (Mandrell *et al.*, 2005; Walker *et al.*, 2002; Wang *et al.*, 1998).

Mass spectrometry of whole cells has been utilised to accomplish two goals, these being either to identify unknown bacteria or to discriminate between species and/or strains. The identification of unknown bacteria using SIBIs or spectral fingerprints has become widespread. Detection of *E. coli*, *Shigella flexneri* and *Aeromonas hydrophila* biomarker ions using MS directly from contaminated water, lettuce or cloth samples, exemplified the scope of the technique and showed that pre-culturing may not even be required (Holland *et al.*, 2000). Whole cell MS has thus unsurprisingly been enthusiastically applied to the fields of healthcare and food-borne infections, with the development of databases permitting rapid identification of test samples compared to reference profiles (Bright *et al.*, 2002). A freely accessible online database of human food pathogens, such as *E. coli* O157:H7, has, for example, been established as an important monitoring tool,

allowing rapid differentiation of pathogenic from non-pathogenic species which may help prevent the spread of food borne infections (Mazzeo *et al.*, 2006). Clearly, the limitation of any database is the range and robustness of the reference spectra (van Veen *et al.*, 2010); as this increases, identification of unknown samples will improve. This was demonstrated by the use of diverse *S. agalactiae* strains from human infections to create a reference database from which greater identification of unknown strains at the species level was achieved compared to the identifications made from a database comprising only reference strains (Lartigue *et al.*, 2009).

Variation in mass profiles between different species as well as strains of the same species, were found even in the earliest ICMS experiments (Claydon *et al.*, 1996; Haag *et al.*, 1998; Krishnamurthy and Ross, 1996; Krishnamurthy *et al.*, 1996). Later, closely related species of *Campylobacter* (Mandrell *et al.*, 2005), β–haemolytic streptococci (Kumar *et al.*, 2004) and *Aeromonas* (Donohue *et al.*, 2006) were differentiated based upon species specific biomarker ions, which, despite some obvious strain differences, were conserved amongst multiple strains of the same species. Discrimination of closely related *Vibrio* species was comparable to that achieved by gene sequencing (Dieckmann *et al.*, 2009) and ICMS of mutans streptococci permitted re-classification (as confirmed by 16S rRNA sequencing) of three strains originally classified incorrectly using traditional methods (Rupf *et al.*, 2005). Phylogenetic relationships between *Arthrobacter* species was also permitted using MS of whole cells, and the resulting dendrogram corresponded almost exactly with the tree produced based upon the 16S rRNA gene sequences (Vargha *et al.*, 2006). Whole cell MS has also provided a suitable alternative method for the speciation of fastidious *Mycobacterium*, for which identification using standard protocols is difficult (Hettick *et al.*, 2006; Pignone *et al.*, 2006). Several researchers have also utilised ICMS for rapid differentiation of methicillin resistant *S. aureus* (MRSA) from antibiotic sensitive strains (Jackson *et al.*, 2005; Walker *et al.*, 2002). Whilst no general MRSA profile could be identified, not only was discrimination from sensitive strains possible, but clonal MRSA isolates with indistinguishable PFGE or phage types were similarly indistinguishable by ICMS (Bernardo *et al.*, 2002; Edwards-Jones *et al.*, 2000). Outbreak strains of *S. pneumoniae* which had been shown using MLST and PFGE to be clonal, were similarly shown using MS of whole cells to be highly related (Williamson *et al.*, 2008).

The use of MS for bacterial typing has many advantages over more routinely used genome-based analytical protocols; the most obvious being that the method is simple, quick and cheap (notwithstanding the initial cost associated with the purchase of the

mass spectrometer itself). This provides the potential for very high sample throughput whilst maintaining high confidence in results by inclusion of several replicates. Additionally, spectrometry of whole cells avoids the reliance upon just a limited number of genes from the entire genome to infer homology, instead analysing all exposed proteins. The requirement for minimal sample preparation has resulted in ICMS being widely utilised for rapid bacterial diagnosis which may increase the speed and efficiency of subsequent treatment and control measures. No studies were found where MS had been utilised for the identification or speciation of *S. uberis,* or any other bacteria derived from mastitis infections. The main aim of the work described in this chapter was thus to develop an effective whole-cell MS protocol to permit the characterisation of *S. uberis* strains from cases of mastitis, and, subsequently, to compare the discriminatory ability of this simple technique with that of MLST conducted using the same collection of strains (described in **Chapter 3**).

4.2 Results

4.2.1 Preparation of *S. uberis* cells for mass spectrometry

To develop a procedure for whole-cell MS of *S. uberis*, firstly, the effect that different methods for preparing cells imparted upon resulting mass profiles was explored. Chemical and physical techniques which have been utilised in the literature were assessed. Bacteria were cultured on BHI agar plates, and experiments conducted by solubilising 10 colonies of *S. uberis* strain 0140J in 100 μl of the appropriate reagent. The specific protocols used are described in **Chapter 2 (Section 2.19)**. Colonies picked from two separate BHI agar plates were used, and 4 technical replicates were conducted for both biological samples. The use of 4 technical replicates from every biological replicate was, unless otherwise stated, the standard procedure for all subsequent experiments.

Representative spectra produced following MS of *S. uberis* cells prepared using different methods are shown in **Figure 4.1**. Additionally, colonies scraped directly from an agar plate, with no processing, were analysed, as were cells prepared from liquid cultures. No distinctive mass peaks were observed, however, in any of these experiments; hence, the data is not presented, and these protocols were not explored further. Spectra produced following analysis of treated bacterial cells from agar plates, were compared to control spectra containing no bacterial cells to identify artefacts from the preparation processes. Spectra obtained from cells treated with lysozyme or protease inhibitors displayed clear peak suppression, as the only peaks produced were those derived from the reagents themselves. Poor spectra were obtained following suspension of cells in ethanol, whilst insufficient peaks were found on spectra from cells which were boiled prior to MS. Suspending cells in acetonitrile, or lysis of cells using beads (ribolysis), produced mass spectra containing more than 15 distinct, high-intensity peaks, distributed over a mass range of 3,000 to 13,000 Da. Such spectra were considered suitable for differentiating between *S. uberis* strains and thus worthy of further investigation. No peaks were visualised on control spectra for these two preparation methods, confirming that the observed peaks derived from bacterial cells only. A single peak was identified on the mass spectrum obtained from a piece of BHI agar solubilised (as far as was possible) in water, however, this was not seen in spectra from ribolysed or acetonitrile-treated cells, suggesting there was also no contamination from the growth medium on the resulting spectra.

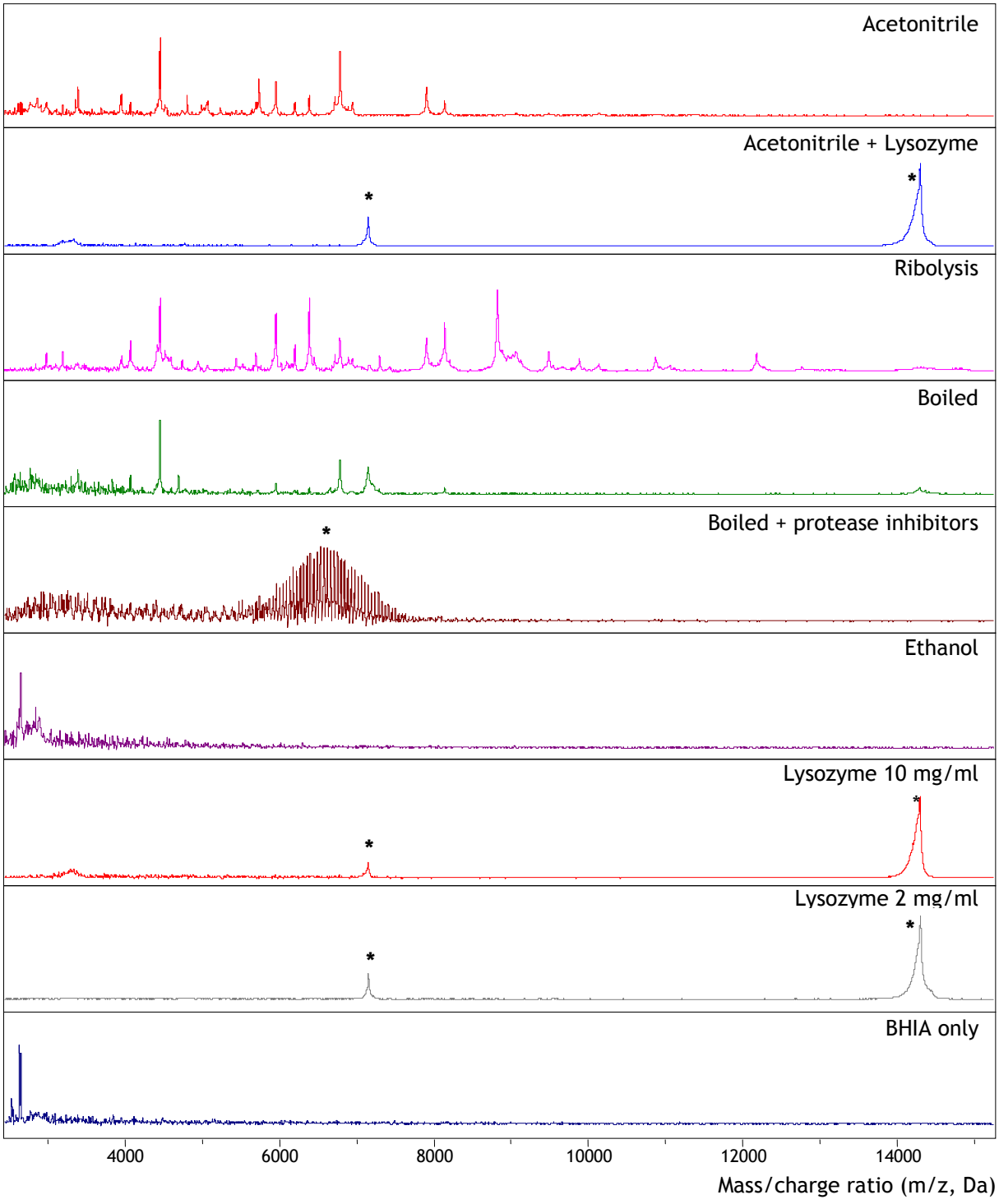


Figure 4.1: Peak profiles produced following disruption of *S. uberis* 0140J cells using various methods. Peaks on profiles containing lysozyme and protease inhibitors (marked with an asterisk) corresponded to artefacts from these reagents, these peaks being similarly produced on profiles from control experiments containing no cells. Ribolysis or acetonitrile treatment produced the best mass spectra and no artefacts were produced. A single peak was identified following MS of a piece of dissolved agar but this was not seen on spectra from cells prepared by ribolysis or with acetonitrile.

Suspension in acetonitrile or ribolysis of cells also produced reproducible mass profiles, as demonstrated by the comparison of spectra from arbitrarily selected biological and technical replicates (**Figures 4.2 & 4.3**). The mass values of five of the largest peaks were annotated onto the spectra to further demonstrate that these values were highly reproducible using both methods, and indeed the peak masses typically only deviated by less than 1 Da between replicates. Two technical replicates from cells which were ribolysed, failed, however, to produce any peaks, and thus could not be included in the comparisons. The mass profiles produced by the two protocols were different, and ribolysing cells resulted in more distinct peaks being identified. This is presumably due to the exposure of proteins from all cell compartments using this method, rather than targeting predominantly those which are extracellular, as is the case when using acetonitrile. The large peak at approx. 4,452 Da was, conserved, however, on *S. uberis* 0140J profiles regardless of the method by which cells were prepared. These two techniques were both suitable for further analysis of *S. uberis* strains as there was no interference from reagents used to prepare cells, results were reproducible and the protocols were quick and easy to perform.

4.2.2 Observations of discrimination between *S. uberis* strains

To determine if mass profiles from ribolysed cells permitted sufficient discrimination between strains of the same species, cells from five *S. uberis* mastitis strains were analysed by MS. Replicates from individual strains were found to be highly reproducible upon visual comparison. Peak conservation was further demonstrated by overlaying mass spectra from all 16 *S. uberis* 0140J replicates, and these were found to match very closely. This was equally observed for all replicates from each remaining strain (data not shown). Some peak differences were readily visualised by eye between distinct strains; the MALDI BioTyper v.1.1 software (Bruker Daltonics, Bremen, Germany) was used to process all replicate spectra from each strain to further explore the differences between the mass profiles produced. Default settings for pre-processing spectra for principal component analysis (PCA) were used. This automated pre-processing normalised and smoothed peaks on mass spectra, as well as reducing background noise to permit more precise comparison prior to PCA. In this analysis the dimensions of the dataset were reduced whilst the main features were retained, reducing the complexity of the data whilst capturing the major variations to allow discrimination between profiles. Peaks within the mass range of 5,000 and 10,000 Da were included in the analysis, where the defined target resolution for the m/z axis was set at 2 Da and the maximum number of principal components to be analysed was limited to 5.

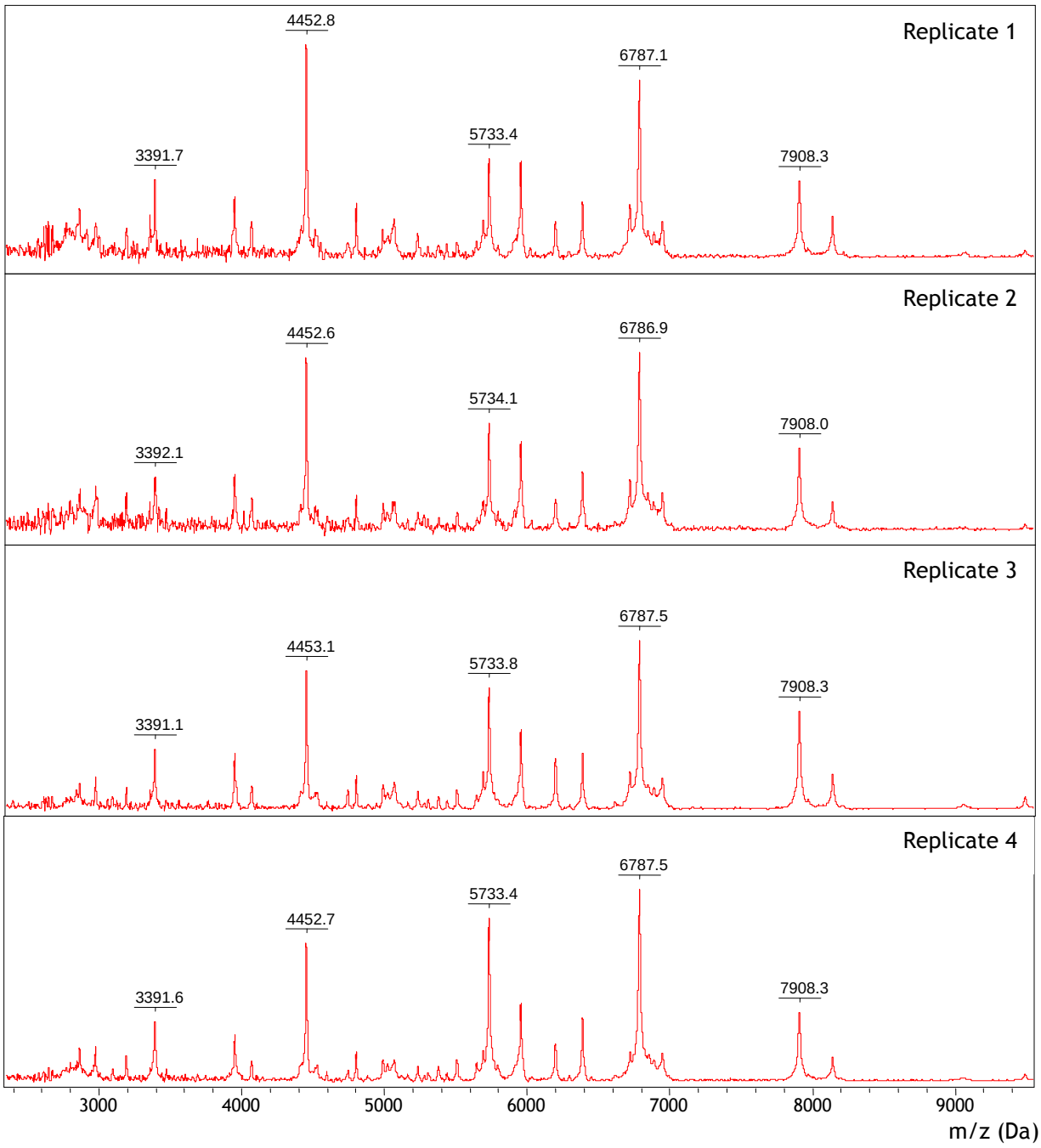


Figure 4.2: Replicate mass spectra obtained from *S. uberis* 0140J following suspension of cells in acetonitrile prior to MS. The precise mass values for the five most prominent peaks are annotated onto the profiles to demonstrate the reproducibility between biological and technical replicates. Typically, a deviation in peak mass of less than 1 Da was seen between replicates.

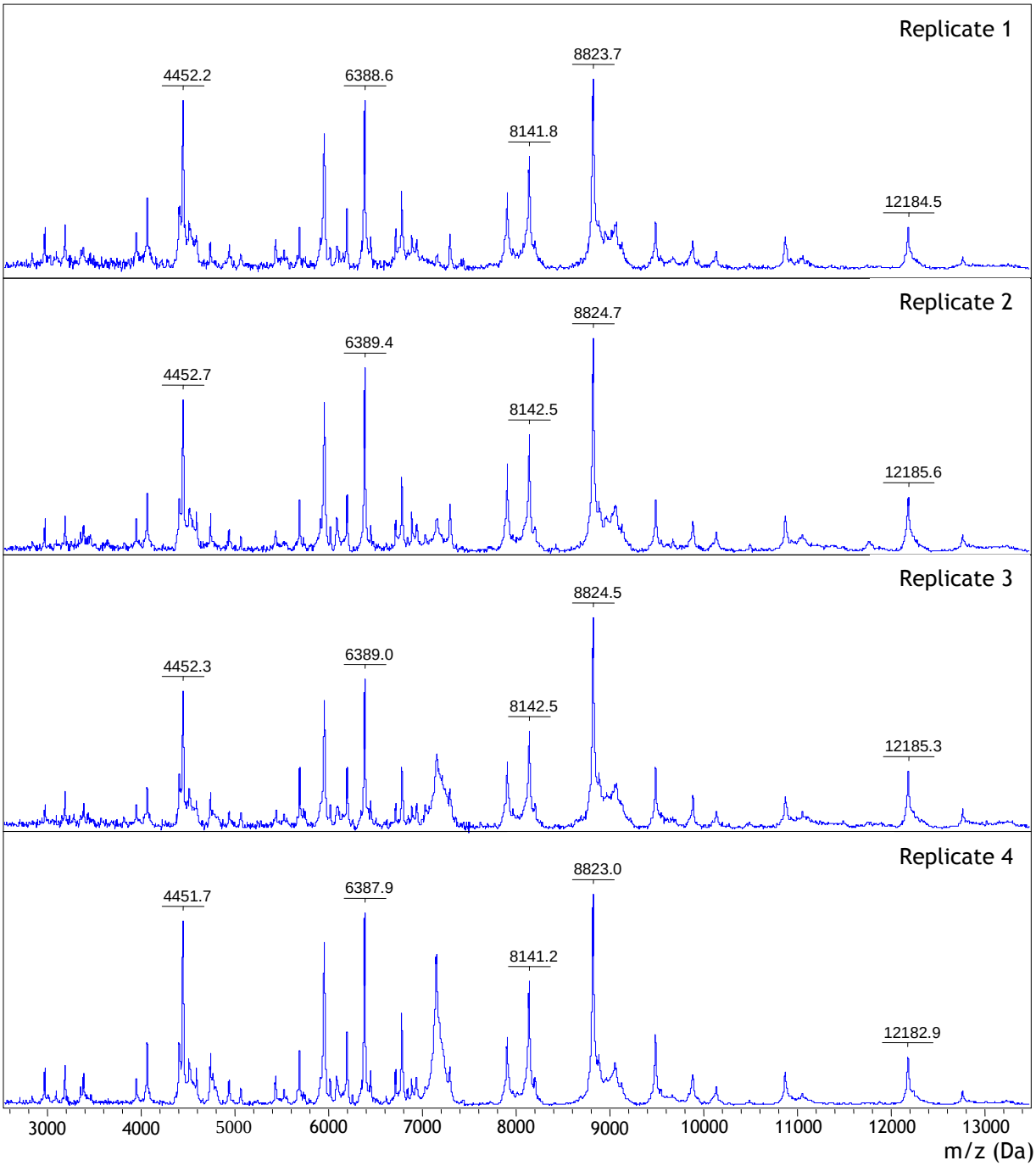


Figure 4.3: Replicate mass spectra produced from *S. uberis* 0140J following ribolysis of cells prior to MS. The precise mass values for five of the most prominent peaks are annotated on the profiles to demonstrate the reproducibility between biological and technical replicates. Typically, a divergence in peak mass of less than 1 Da was seen between replicates.

Quantum clustering (QC), using the computer-defined default settings permitted visualisation of the relationships between spectra based upon the three main principal components identified. Replicate spectra from the same strain generally clustered together very well and were predominantly assigned to a single cluster by the software. As most strains were clustered separately, this suggests that sufficient differences were identified between the mass spectra of these strains to allow discrimination under the defined parameters (data not shown). The analysis placed strains I6 and 1:93 within the same cluster, implying that the mass spectra from these strains were very similar despite these being isolated from different countries and different animal hosts. Although not placed within the same cluster, strains 20569 and T1-3 also seem to be closely related. The potential of MS to discriminate between *S. uberis* mastitis strains using cells prepared by ribolysis was thus clearly demonstrated.

4.2.3 Comparison of mass spectra from different strains using ribolysis or acetonitrile to prepare cells

As successful discrimination between strains was achieved using ribolysed cells from 5 strains, a further experiment was conducted using two biological replicates from nine strains. Included in the analysis were bovine reference strains from the UK (0140J) and Germany (20569), UK bovine mastitis strains T1-3, T1-57, T2-74 and T3-16, a bovine isolate from the US (1:93), and Italian bovine (I22) and ovine (I6) mastitis strains. Cells were prepared either by ribolysis or by suspension in acetonitrile. Visual comparison of replicate spectra from *S. uberis* 0140J, prepared using both methods, showed high reproducibility, both within this experiment and compared to earlier experiments (data not shown). The standard deviation between the mass values of 20 abundant peaks from these replicates was low, typically being less than 2 Da (**Appendix 4**) demonstrating the robustness of both protocols. Despite the two preparation methods disrupting cells differently, approx. half of the abundant peaks identified using both methods had the same mass, suggesting that many of the same proteins had been detected and implying that these are in abundance in the cell.

Representative mass spectra from each of the nine *S. uberis* strains, prepared either by solubilisation in acetonitrile or ribolysis of cells, are compared in **Figures 4.4 and 4.5**. As in earlier experiments, only small differences were visible between the spectra from different strains and more peaks were identified following ribolysis of cells. No peaks were visualised on mass profiles following ribolysis of I22 cells for all but one replicate. In comparison to other strains this remaining spectrum, as well as profiles obtained

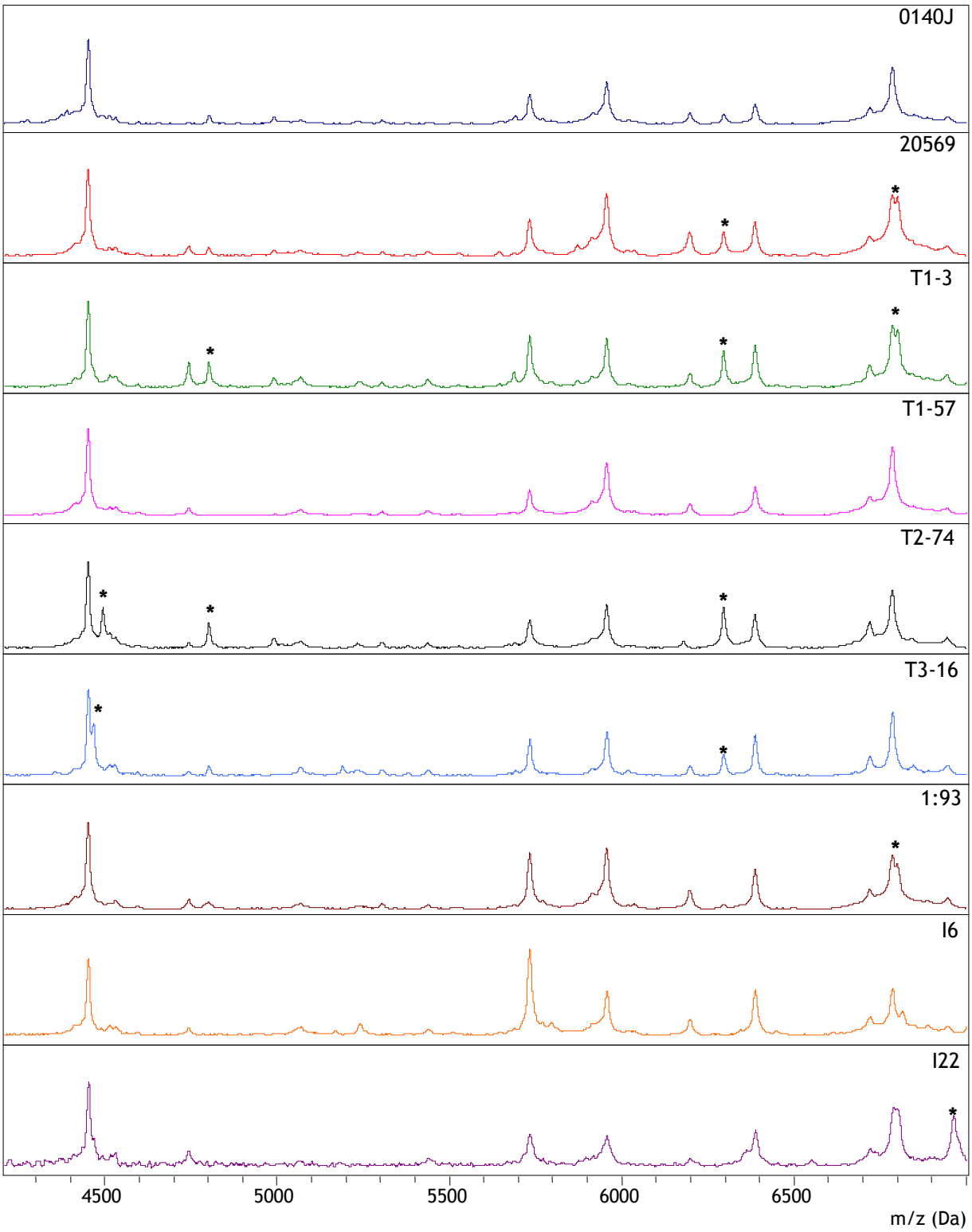


Figure 4.4: Comparison of mass profiles produced following solubilisation of cells from nine *S. uberis* strains in acetonitrile. The most notable peak differences between strains are marked with an asterisk.

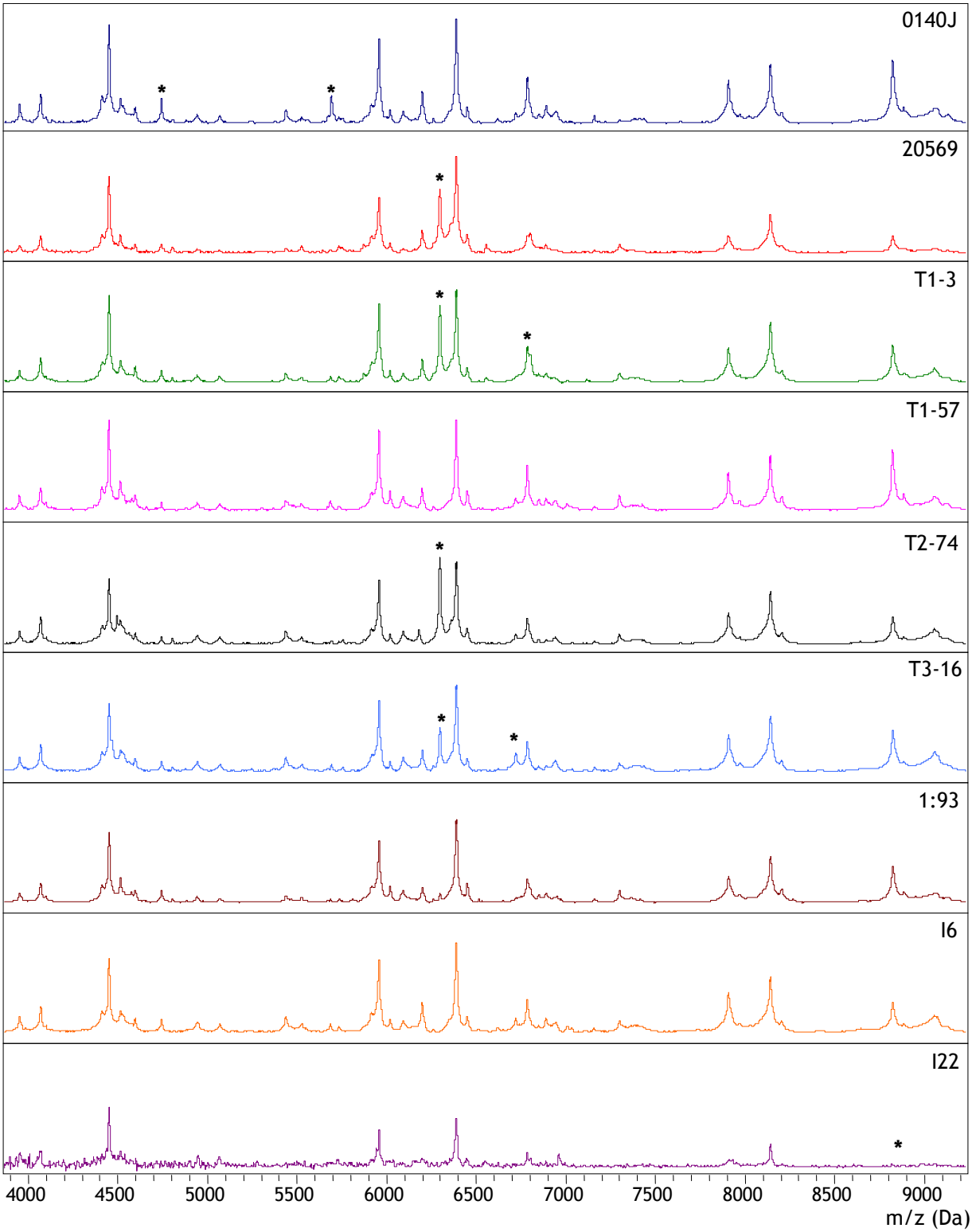


Figure 4.5: Comparison of mass profiles produced following disruption of cells from nine *S. uberis* strains by ribolysis. The most notable peak differences between strains are marked with an asterisk.

following suspension of I22 cells in acetonitrile, displayed fewer, weaker peaks and the background noise was higher than on spectra from other strains.

Automated PCA and QC were completed using all replicate spectra from either acetonitrile or ribolysis derived samples (except those displaying no peaks). Clustering of both data sets placed replicates from one strain within a single, or closely related clusters, and demonstrated relationships between specific strains (**Figure 4.6**). Despite the spectra from ribolysed and acetonitrile-solubilised cells being different, and thus the clustering distinct, the relationships inferred between strains using both techniques were relatively similar. In both analyses, for example, strains 0140J and T1-57 were placed within the same cluster, these strains also being grouped closely with strains I6 and 1:93. Similarly, strains T1-3 and 20569 were closely clustered in both analyses. Interestingly, there was also an association between spectra from strains 0140J, T2-74 and T3-16 when processed using acetonitrile (**Figure 4.6**). This was significant given that these three strains were all representatives of the dominant ST 5 CC identified in the UK by MLST analysis (**Chapter 3**). This relationship was not evident however, when cells were prepared by lysis with beads. Strain 0140J was also, however, closely grouped with additional strains which were not part of the ST 5 CC, implying that whilst there may be some correlation between gene and protein based typing methods, this was by no means definitive and clearly requires further research. It was again confirmed however, that despite apparently minimal visual differences between mass spectra, sufficient variation was exhibited to permit discrimination between different strains using the BioTyper software.

In an additional experiment, BHI agar plates prepared with 200 μM of the chelating agent 2, 2'-dipyridyl were used to culture cells for MS. Following preparation of cells by ribolysis or solubilisation in acetonitrile, no visible differences were observed between the mass profiles obtained from cells grown on restricted plates and those from BHI agar plates. Clustering analysis produced similar results to those obtained using colonies from BHI agar plates, although clusters were generally not as well defined (data not shown). The use of restricted plates for whole cell MS was thus not explored further.

The aim of this study was to develop a rapid, high-throughput typing protocol. Consequently, because preparing cells by ribolysis was significantly more labour intensive than solubilisation in acetonitrile, but similar clustering results were achieved using both techniques, acetonitrile was adopted as the more favourable preparation method for further experiments to explore in more detail the potential of MS for typing

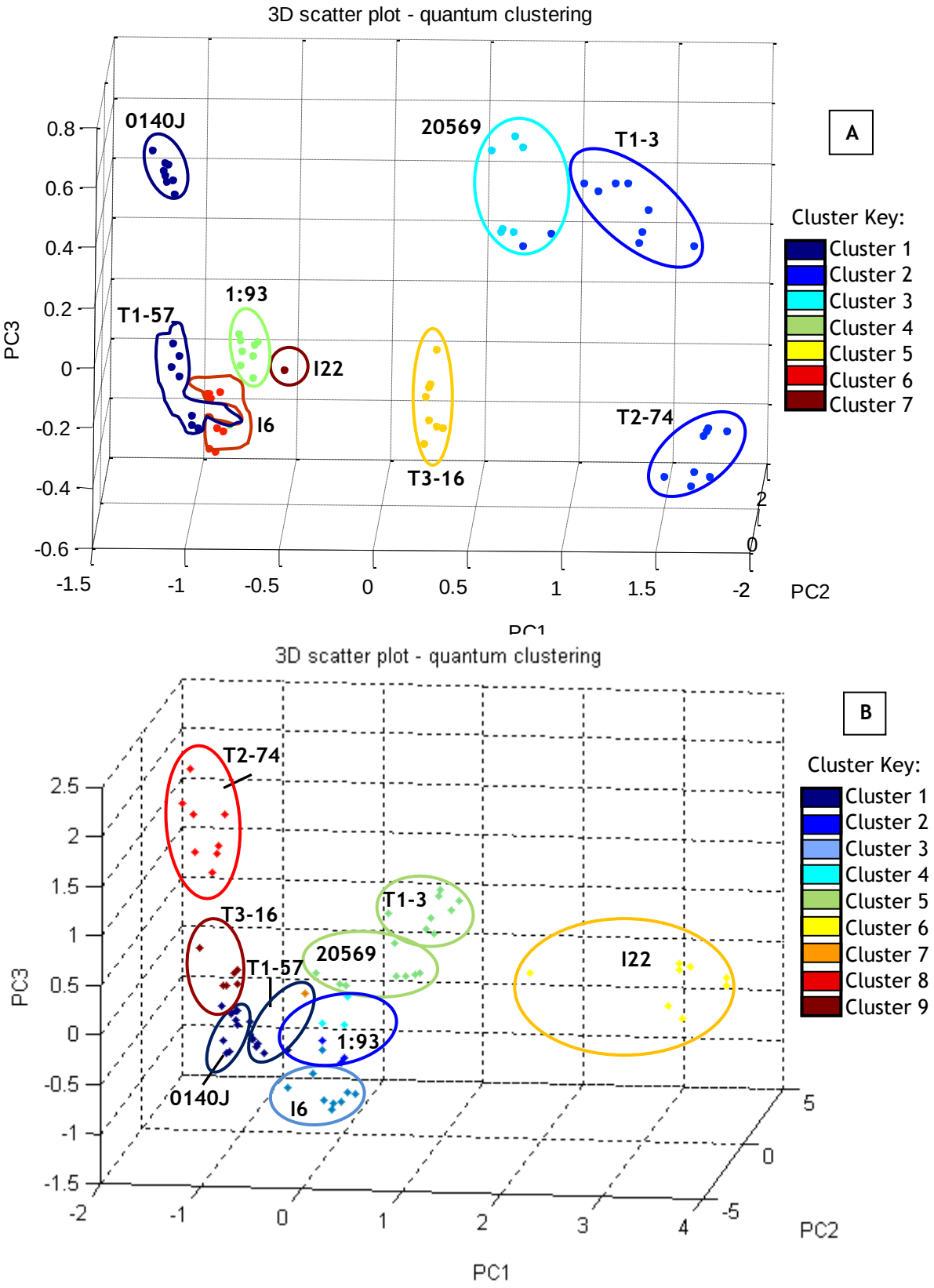


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Figure 4.6: Quantum clustering of processed mass spectra from nine *S. uberis* mastitis strains obtained following ribolysis of cells (A) or suspension of cells in acetonitrile (B).

A) Spectra were batch pre-processed using the default settings for PCA including peaks within the mass range of 5,000 and 10,000 Da. The PCA was conducted to include peaks from the same mass range with a resolution of 2 Da and a maximum of nine principal components were considered. Default settings were used for QC and seven clusters were identified from the mass data.

B) Spectra were batch pre-processed using the default settings for PCA including peaks within the mass range of 4,000 and 10,000 Da with a resolution of 1 Da, and selecting the maximum number of peaks as 15 with a threshold value of 0.1, so that only peaks with an intensity of at least 1 % of the highest peak were included to reduce background noise. The PCA was conducted to include peaks from the same mass range with a resolution of 2 Da and a maximum of nine principal components were considered. Default settings were used for QC and nine clusters were identified from the mass data.

Replicate spectra from individual strains are grouped within user drawn circles to demonstrate reproducibility. Differences between strains are visualised by the clustering based upon the variation within the first 3 principal components identified by the software. Generally, clustering of replicates was also confined to one computer derived cluster, where this was not seen, clusters were closely grouped. In both analyses strains 0140J and T1-57 were grouped closely together, as were 20569 and T1-3.

S. uberis mastitis isolates.

4.2.4 Characterisation of Italian mastitis isolates by MS

4.2.4.1 Preparation of isolates using acetonitrile

Following the success of the previous experiment, the entire collection of 50 Italian ovine and bovine *S. uberis* mastitis isolates (**Table 3.6**) were analysed by suspending colonies in acetonitrile prior to MS. During sample preparation it was noted, however, that many of the Italian isolates produced extremely mucoid colonies on BHI agar plates. These mucoid colonies, unlike those from the remaining isolates, could not be effectively solubilised in acetonitrile to produce an opaque mixture. The mucoid nature of these colonies was attributed to the production of hyaluronic acid capsule around the cell (described in **Section 1.4.3**).

The species origin or multi-locus ST did not appear to be closely related to observations of excessive capsule production (as defined by insolubility in acetonitrile). Of the encapsulated isolates, 38 % were of ovine and 62 % were of bovine origin. Both isolates assigned to the ST 86 CC were, however, found to be capsular, whilst none of the isolates belonging to the ST 5 or ST 143 CCs (n=5) were represented. Furthermore, all genetically closely related isolates placed into BURST groups 1 (n=4) and 3 (n=2), as determined in **Chapter 3**, were capsular. These capsular isolates were also subsequently found to be poorly ionised, producing unsuitable mass spectra (**Figure 4.7**). Interestingly, isolate I22, from which poor spectra were produced in the previous experiment, was one of these isolates. A single peak between 2,600 and 2,800 Da dominated the mass profiles from all highly mucoid colonies. The poor spectra may have resulted from ionisation of only the capsular material during MS, or, as a result of the capsule physically preventing ionisation of additional proteins from the cell. Alternatively, ions derived from the capsule may have been present in such high abundance that the visualisation of additional lower intensity ions, which may still have been produced, was suppressed. Considering that 26 % of the Italian collection were highly mucoid on this occasion, and thus poorly ionised under these conditions, comparative analysis of all the Italian isolates was not conducted at this stage.

4.2.4.2 Preparation of isolates using hyaluronidase and acetonitrile

To overcome the problems caused by the hyaluronic acid capsule interfering with mass spectrometric analysis, an additional treatment step was included. Hyaluronidase was utilised to remove capsular material from *S. uberis* cells prior to suspension in

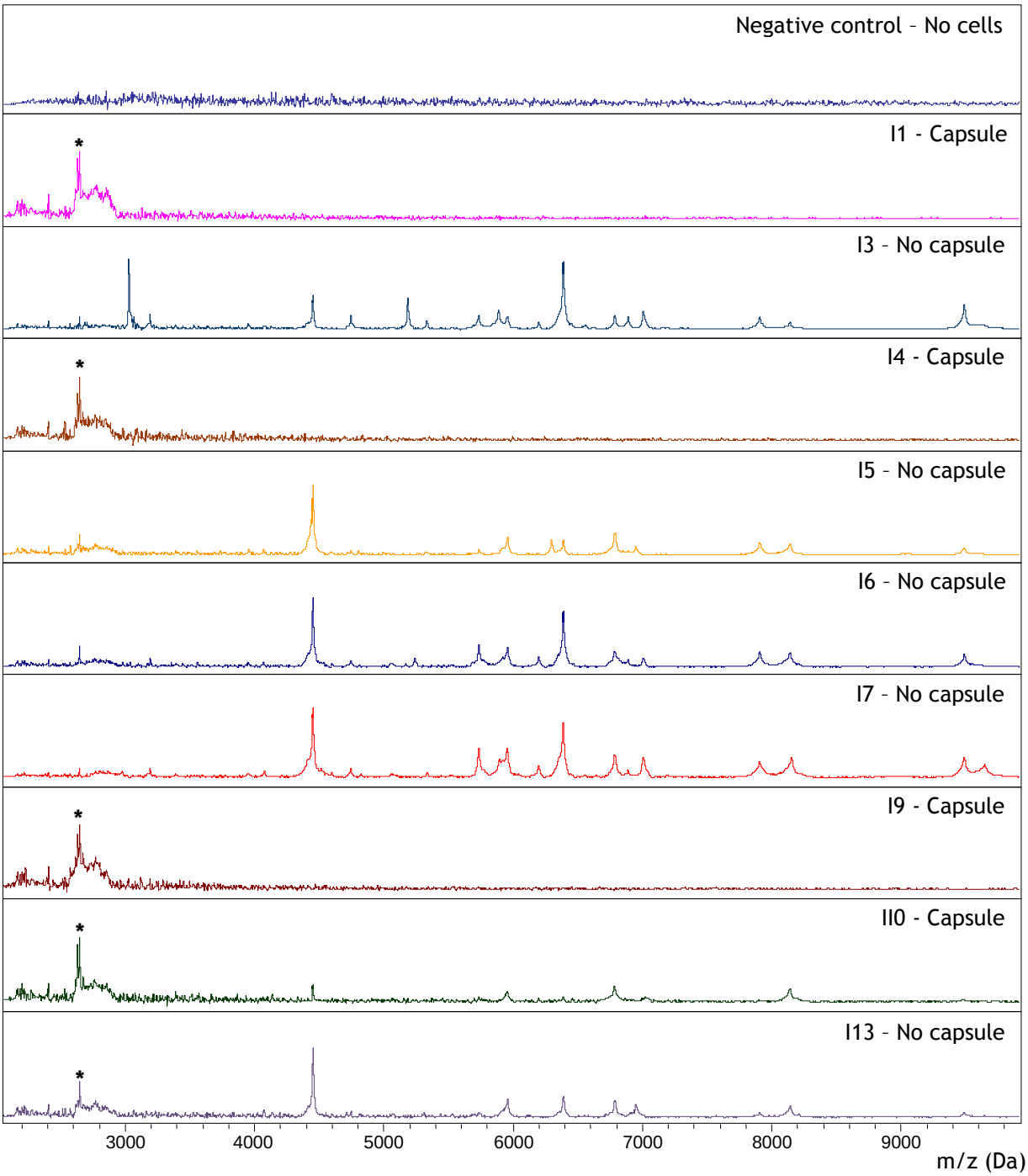

Figure 4.7: Mass profiles produced from *S. uberis* isolates demonstrating interference caused by excessive capsule production. The single peak visible on spectra from cells which were not completely solubilised in acetonitrile (and thus presumably capsular) is marked with an asterisk and is likely to be derived from ionisation of capsular material which has prevented or repressed ionisation of additional proteins. Clearly isolate I13, which was at least partly solubilised in acetonitrile, also produced capsule to a lesser extent as a small peak between 2,600 and 2,800 Da is visible and the remaining peaks in the mass profile are poor.

acetonitrile. Italian isolates which had been found to be either highly or weakly encapsulated (I9 and I12 respectively) were used to test this protocol. For optimisation of the protocol, different conditions (incubation at RT for 1 h or at 37 °C for 1 or 2 h) and final hyaluronidase concentrations (25, 50 or 75 μg/ml) were tested. To remove hyaluronidase prior to MS, cells were also washed once, twice or thrice with distilled water. Cells were then re-suspended in acetonitrile as previously and subjected to MS.

Mass spectra produced from weakly encapsulated isolate I12 were very similar regardless of the test conditions, and as such minimal variation was visualised between all 162 spectra obtained (data not shown). The effect of additional washes was limited, but diluted samples slightly, such that two washes standardised the results somewhat and reduced background, whilst 3 washes diluted the features to an extent that background interference was increased (data not shown). The effect of increasing the hyaluronidase concentration and varying the incubation conditions, on the mass profiles obtained from encapsulated *S. uberis* isolate I9 are shown in **Figure 4.8**. All conditions identified the same prominent peaks, being a vast improvement on the earlier profile produced without hyaluronidase (**Figure 4.7**). The mass spectrum produced following incubation for 2 h at 37 °C with 50 μg/ml hyaluronidase was however superior, yielding the clearest, smoothest peaks and the lowest background noise. Replicate mass spectra from isolate I9, produced under these optimal conditions, were visually compared and found to be highly reproducible, whilst also differing from the profiles obtained from the acapsular isolate I12 (**Figure 4.9**). This protocol was thus considered suitable for further analysis of Italian mastitis isolates.

4.2.4.3 Profiling isolates using hyaluronidase and acetonitrile

Four biological replicates were prepared for each of 50 Italian *S. uberis* isolates, and prior to solubilisation in acetonitrile, cells were incubated with 50 μg/ml hyaluronidase for 2 h at 37 °C and then washed twice with water. BioTyper software was used to analyse mass spectra.

Pseudo-gel images were used to visualise peak differences between processed mass spectra from isolates which were assigned to user-defined groups. Firstly, grouping isolates based upon host origin demonstrated that while several peaks were present in most or all isolates, no peaks appeared to be unique to either all ovine or all bovine isolates (data not shown). Alternatively, grouping isolates by their multi-locus ST and assigned BURST group (BCC) demonstrated that some peaks were either conserved or

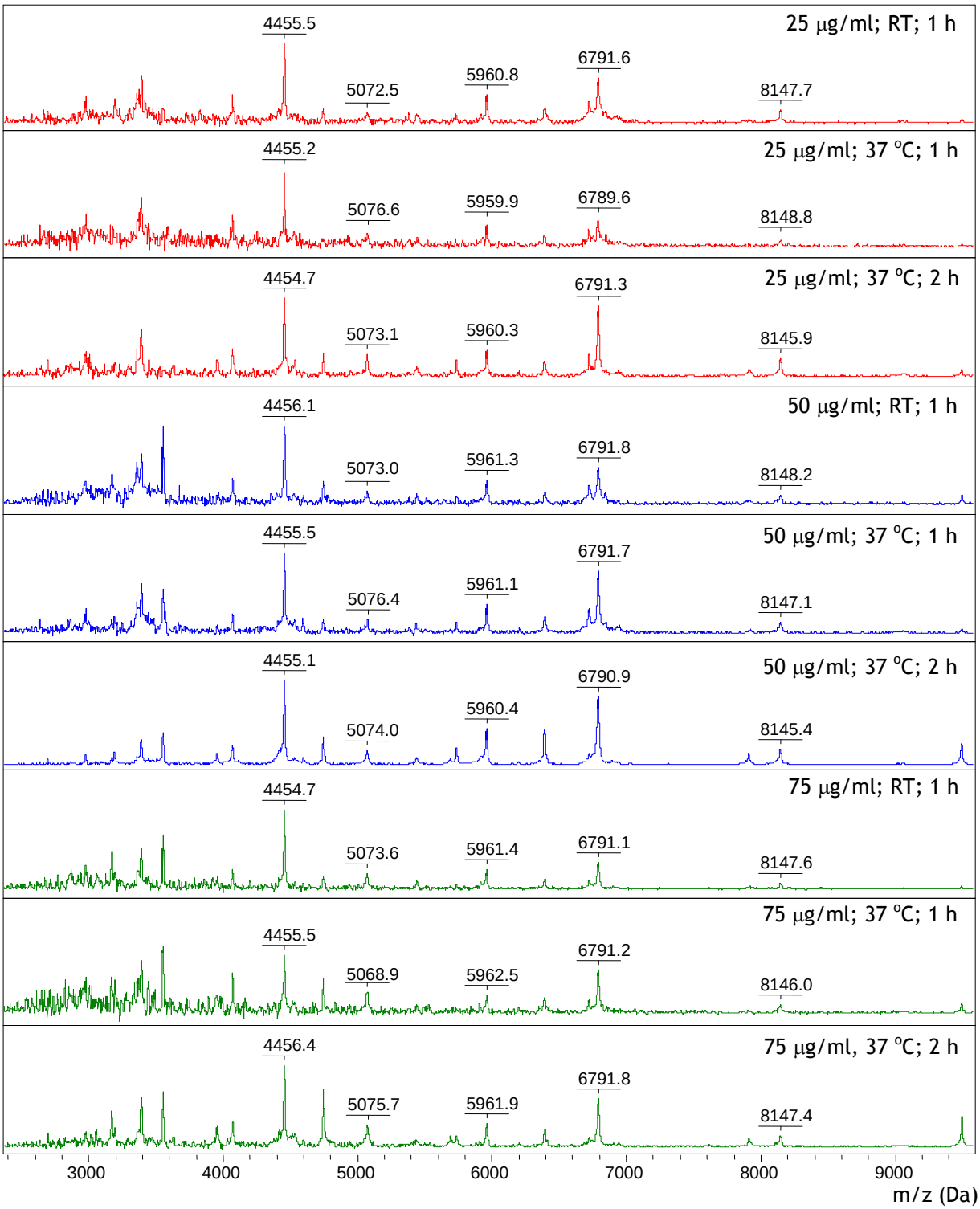

Figure 4.8: Impact of hyaluronidase treatment on mass spectra from highly capsular *S. uberis* I9 washed twice with water prior to MS. The same major peaks were clearly visible in all spectra, but, incubation of cells with 50 µg/ml hyaluronidase for 2 h at 37 °C prior to solubilisation in acetonitrile produced the most optimal profile, with sharper and smoother peaks and less background noise than all other profiles.

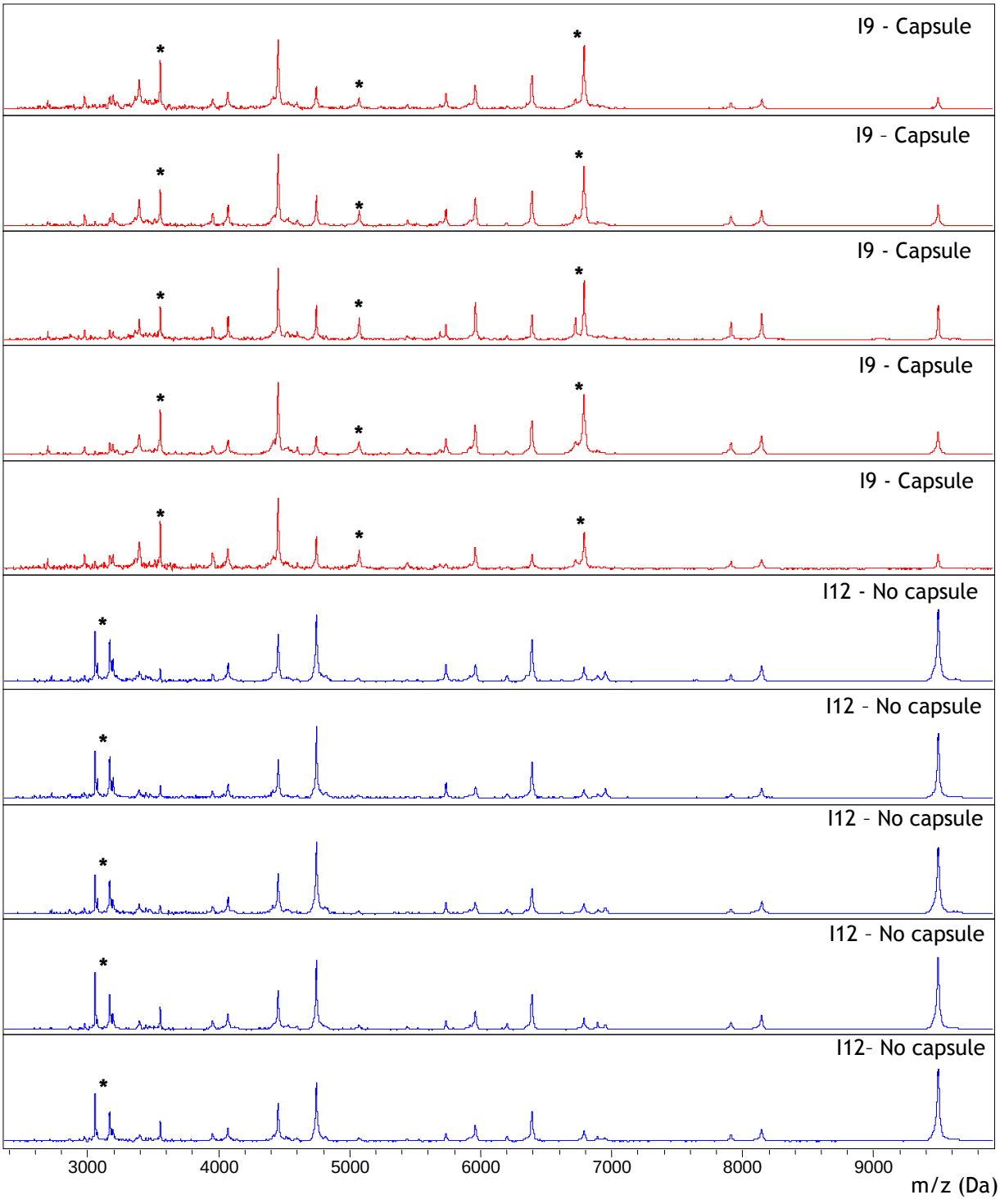

Figure 4.9: Reproducibility of mass spectra obtained from *S. uberis* isolates following hyaluronidase treatment prior to solubilisation in acetonitrile. Cells were incubated in 50 μg/ml hyaluronidase for 2 h at 37 °C, then washed twice with dH_20 and re-suspended in 100 μl acetonitrile. Capsular I9 and acapsular I12 are shown in red and blue respectively. Replicates from the same isolate were highly reproducible whilst clear differences were visualised between spectra from the two different isolates. Distinctive peak differences between the isolates are marked (*).

absent amongst all isolates of a ST or group (**Figure 4.10 A**). Equivalent peaks were, for example, absent amongst all replicates from isolates representing ST 305 (I36, I37, I40 and I47), although these were also absent in I29 profiles which was not a representative of this ST. Similarly, all replicates from isolates representing ST 293 (I2 and I8) lacked distinctive peaks as well as I3 (which was unrelated to ST 293). Finally, no peaks were specific to an individual Italian region from which the samples were isolated, although some peaks were conserved or absent within individual farms (**Figure 4.10 B**). All replicates of isolates I35, I36, I38, I40, I41 and I47 distinctly lacked four peaks and these, with the exception of I41, were isolated from a single farm. Similarly, replicates from isolates I41, I42 and I43 from the same farm, all shared a characteristic peak; these isolates also shared the same ST. Demonstration of some correlation between mass spectra and isolate source or multi-locus ST has thus been demonstrated, although this was by no means definitive. Despite significant processing of data, clustering analysis failed to identify obvious groups. In many cases, replicates from individual isolates were distributed amongst several clusters (data not shown). No obvious relationships between or within clusters could be identified.

4.2.4.4 Profiling Italian isolates using ribolysis and acetonitrile

As bacterial capsule production interfered with mass profiles, and clustering of replicate spectra from cells treated with hyaluronidase was not particularly reproducible, ribolysis was reconsidered to be a suitable alternative method for breaking open cells prior to solubilisation in acetonitrile. This protocol was trialled using one highly and one weakly encapsulated isolate. The amended protocol produced mass spectra from both isolates which appeared to be suitable for further analysis. In earlier experiments, peaks on some profiles were quite faint, so to try and rectify this problem a larger number of colonies were used for ribolysis and subsequent solubilisation in acetonitrile. Increasing the number of colonies used to 20 only had a marginal impact on the profiles from the two tested isolates, although it did appear to improve the profile from the encapsulated strain very slightly (data not shown).

As in previous experiments four biological replicates were conducted for each of the fifty Italian isolates, but in this experiment 20 colonies were used per replicate instead of 10. Unfortunately, upon subsequent analysis, some poor spectra were still produced, where the intensity of the background peaks were greater than 10 % that of the highest peak. To avoid these weak profiles interfering with the clustering analysis, these technical replicates were discarded. Isolates for which more than two replicate spectra were disregarded were: I1 (13 reps), I37 (11 reps), I41 (9 reps), I9 and I22 (7 reps), I3

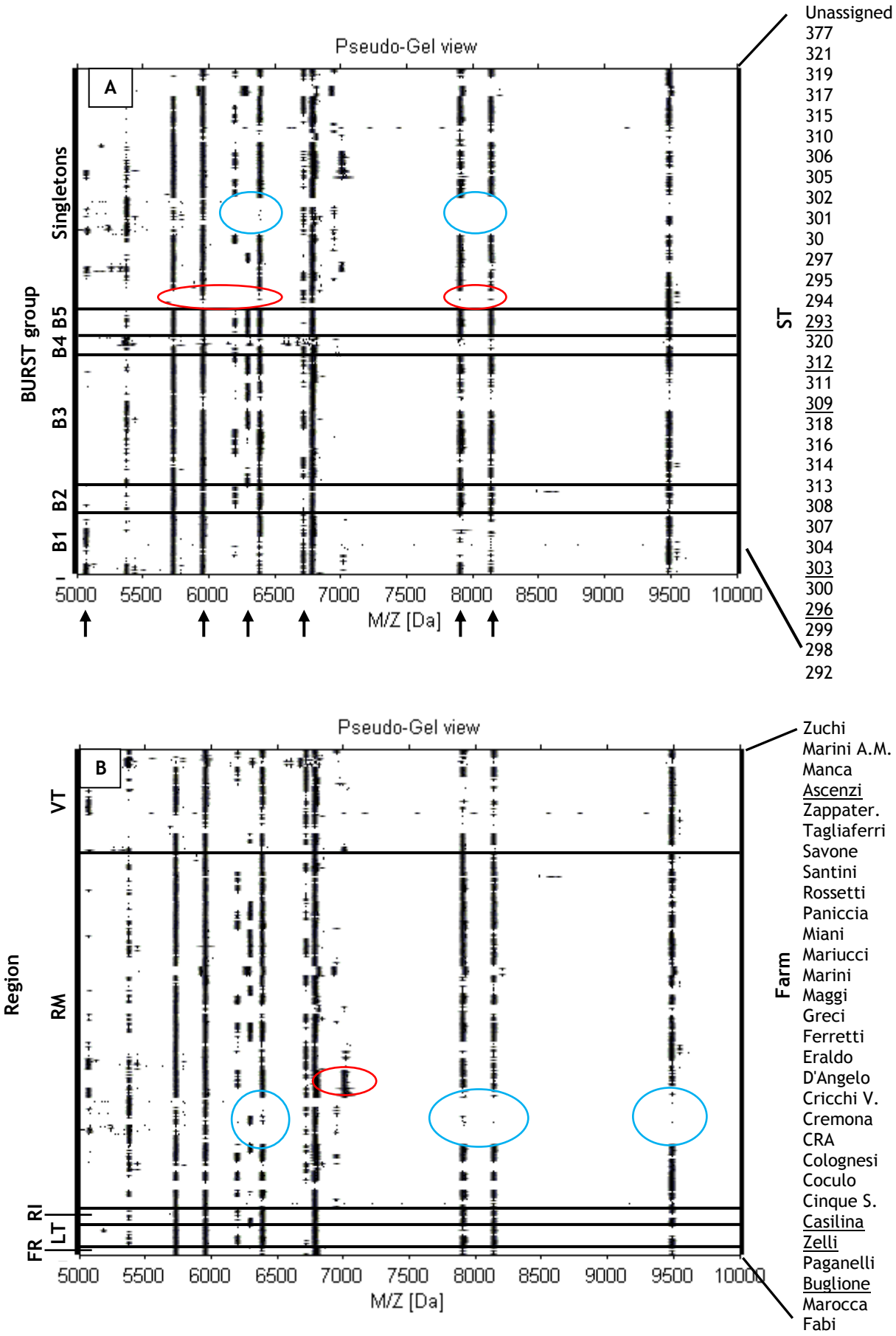

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Figure 4.10: Pseudo-gel images of processed mass spectra from Italian *S. uberis* mastitis isolates arranged by multi-locus ST and BURST group (A) or farm and region from which they were isolated (B). Mass spectra were batch processed to include peaks within the mass range of 5,000 and 10,000 Da with a resolution of 1 Da. The maximum number of peaks to be included was 10, which, to reduce background noise, had an intensity of at least 1.5 % that of the highest peak.

A) Isolates were grouped by multi-locus ST and BURST group (B1 to B5 or unassigned singletons). Presence or absence of specific peaks (marked with arrows) was similar within BURST groups. Replicates from isolates I29, I36, I37, I40 and I47 all shared four distinctly absent peaks (circled blue). Interestingly, these isolates with the exception of I29 represented the same ST (ST 305). Similarly isolates I2, I3 and I8 all lacked similar peaks (circled in red), with I2 and I8 also being representatives of the same ST (ST 293).

B) Isolates were grouped by farm and region. Replicates from isolates I35, I36, I38, I40, I41 and I47 all shared four distinctly absent peaks (circled blue). Interestingly all these isolates, except I41, were isolated from the same farm. Similarly replicates of isolates I41, I42 and I43 all displayed a characteristic peak (circled in red) and all these isolates originated from a single farm and represented a single ST (ST 319).

and I4 (6 reps), I49 (4 reps) and I17 and I19 (3 reps). It cannot be considered a coincidence that isolates previously found to be highly encapsulated (based on insolubility in acetonitrile) were responsible for 75 % of the technical replicates also discarded in this experiment. In the remaining 11 cases, only one or two replicates were removed from the analysis for each isolate and no isolate had to be completely disregarded from subsequent analysis.

Automated PCA and QC were conducted on processed mass spectra and identified nine clusters within the dataset. The settings used for PCA included peaks within the mass range of 5,000 and 10,000 Da, with the maximum number of peaks to be included set to 20 which also exhibited an intensity of at least 1.5 % that of the highest peak. The clustering of individual replicates from each isolate was determined, and generally most replicates from the same isolate were assigned to one cluster (**Table 4.1**). It was seen in fact, that for 86 % of the isolates, at least 60 % of the corresponding replicate spectra were placed within one cluster. Notably, cluster 4 was comprised of 9 isolates, all of which retained the complete set of 20 replicates producing optimal spectra; and for seven of these isolates, 100 % of the corresponding replicates were assigned to this cluster only. This suggests that the profiles representative of this cluster are very distinct, and that the isolates in this cluster are closely related in terms of their protein profiles. Included in this cluster were both isolates representing ST 316 which were also derived from the same farm, but no obvious additional link could be found between the remaining isolates in this cluster.

Cluster 9 comprised just two ovine isolates, I2 and I8, which were derived from the same farm and also represented the same ST. None of the replicate spectra from these isolates needed to be removed from analysis and all the replicates were placed within this cluster. Furthermore, no replicates from any other isolates were assigned to this cluster. Again it is clear that the profiles from these isolates are notably distinct from the remaining isolates. Clusters 5, 6 and 7 all comprised just a single isolate, but again all replicates were assigned to these clusters only. Isolate I34, which was not assigned a ST following MLST due to the absence of the *yqiL* gene, was placed into cluster 7, whilst isolate I24 formed cluster 5 and was not grouped with isolate I22 with which it was found to be most closely related by BURST analysis. Isolate I33 was also grouped individually into cluster 6. This isolate was not typed by MLST, as gDNA could not be extracted in sufficient concentration and purity due to poor growth in liquid medium. All isolates assigned to BURST group 1 were represented in cluster 3, as were 9 of the 13 highly capsular isolates (**Section 4.2.4.1**).

Table 4.1: Cluster assignment for replicate mass spectra from Italian *S. uberis* isolates.

Isolate	No. Reps [A]	Major cluster (F %) [B]	Additional clusters (F %) [C]	Farm	ST (BCC) [D]
I3	54	1 (83)	2 (13), 7 (2) & 8 (2)	Zappaterreno	294
I7	20	1 (60)	2 (35) & 3 (5)	Unknown	297
I18	19	1 (68)	2 (32)	Coculo	305
I44	19	1 (53)	2 (47)	Paganelli	320 (5)
I6	19	2 (47)	1 (32) & 3 (21)	Ferretti	377
I12	20	2 (80)	3 (15) & 1 (5)	Tagliaferri	300 (2)
I13	20	2 (75)	3 (15) & 1 (10)	Miani	301
I14	20	2 (70)	1 (25) & 3 (5)	Tagliaferri	Unassigned
I15	20	2 (65)	3 (25) & 1 (10)	CRA	302
I21	20	2 (80)	1 (20)	CRA	308 (3)
I27	20	2 (80)	1 (20)	Santini	313 (3)
I29	20	2 (70)	1 (30)	Zelli	315
I32	20	2 (75)	1 (25)	Marocca	317
I35	18	2 (39) & 4 (39)	3 (22)	Colognesi	318 (3)
I36	20	2 (60)	1 (25) & 3 (15)	Colognesi	305
I38	19	2 (74)	1 (21) & 3 (5)	Colognesi	318 (3)
I40	60	2 (63)	1 (18) & 3 (18)	Colognesi	305
I46	19	2 (63)	4 (26), 1 (5) & 3 (5)	D'Angelo	312 (5)
I47	19	2 (63)	1 (26) & 3 (11)	Colognesi	305
I48	20	2 (60)	1 (40)	Coculo	307 (3)
I50	20	2 (75)	1 (25)	Buglione	321
I1	7	3 (100)	-	Cricchi Valerio	292 (1)
I4	14	3 (64)	2 (36)	Eraldo	295
I9	14	3 (86)	1 (7) & 2 (7)	Manca	298 (1)
I10	18	3 (100)	-	Cricchi Valerio	299 (1)
I11	20	3 (95)	4 (5)	Cricchi Valerio	299 (1)
I17	17	3 (100)	-	Marini	304 (3)
I19	17	3 (59)	1 (12) & 2 (29)	Casilina	306
I22	13	3 (54)	2 (23), 1 (15) & 4 (8)	Marini A. M.	309 (4)
I23	20	3 (80)	2 (15) & 1 (5)	Marini A. M.	310
I26	20	3 (45)	1 (40) & 2 (15)	Greci	304 (3)
I37	9	3 (100)	-	Cinque Stelle	305
I41	11	3 (82)	8 (18)	Cremona	319
I49	16	3 (94)	1 (6)	Zuchi	Unassigned
I5	20	4 (100)	-	Rossetti	296 (2)
I16	20	4 (100)	-	Ascenzi	303 (3)
I20	20	4 (75)	3 (25)	Fabi	307 (3)
I25	20	4 (100)	-	D'Angelo	312 (5)
I28	20	4 (100)	-	Santini	314 (3)
I30	20	4 (100)	-	Paniccia	316 (3)
I31	20	4 (100)	-	Paniccia	316 (3)

I39	20	4 (100)	-	Savone	30
I45	20	4 (60)	3 (30) & 2 (10)	Maggi	318 (3)
I24	18	5 (100)	-	Marini A. M.	311 (4)
I33	18	6 (100)	-	Mariucci	ND
I34	20	7 (100)	-	CRA	Unassigned
I42	20	8 (75)	3 (15) & 2 (10)	Cremona	319
I43	19	8 (53)	3 (21), 2 (16) & 1 (11)	Cremona	319
I8	20	9 (100)	-	Manca	293
I2	20	9 (100)	-	Manca	293

Frequency (F) values in brackets represent the percentage of the replicates from that isolate which were assigned to the cluster in question.

[A] Number of replicates (Reps) included for analysis (i.e. those with suitable mass profiles).

[B] Major cluster identified.

[C] Additional clusters to which replicates were assigned.

[D] Sequence type and BURST CC (BCC) assignment for each isolate, as determined by MLST (**Chapter 3**). For isolate I33 MLST was not done (ND), whilst isolates I14, I34 and I49 were not assigned STs as the *yqiL* gene could not be amplified from these isolates.

For cases where more than one isolate represented one ST, the clustering obtained by BioTyping for these isolates is highlighted in **Table 4.2**. The majority of replicate spectra from isolates I42 and I43 (ST 319) were placed into cluster 8, whilst isolate I41 (which was also ST 319) was placed in cluster 3, although 18 % of replicates were also assigned to cluster 8. Also of interest was the placement of isolates I35 and I38 (ST 318) into cluster 2 whilst I45 (also ST 318) was placed into cluster 4. Interestingly, isolates I35 and I38 originated from the same farm, whilst I45 was from a distinct farm. Similarly, three isolates representative of ST 305 from the same farm were placed into cluster 2, whilst the fourth and fifth isolates representative of ST 305, which originated from different farms, were placed into different clusters. It was also seen that all isolates from one farm (Colognesi) were clustered together despite representing different STs.

Typical mass spectra representing each of the nine BioTyper clusters are shown in **Figure 4.11**. Some clear differences can be visualised, but there is a high degree of similarity between profiles from clusters 1, 2, 3, 5 and 6. Isolates from clusters 7 and 9 were either very poorly disrupted by ribolysis, or ion suppression by the high abundance of the protein responsible for the peak at 3,000 Da impacted upon resulting profiles. Interestingly, a region between 4,450 and 5,070 Da on the representative profile from cluster 4 appears very distinctive and may explain why this cluster was so well resolved. Similarly, a distinctive peak at 7,000 Da was identified on the representative mass profile for cluster 8.

A dendrogram was generated to visualise the calculated distances between isolates based upon mass profiles. A main spectra database was created for this purpose where all replicates from each isolate were loaded together. The BioTyper programme then created a 'main spectrum' of peaks characteristic for that isolate from all the replicate spectra available. The main spectra of different isolates were then compared, and, using the default settings of the software, this analysis generated a dendrogram of the relatedness of the 50 Italian isolates based upon their mass profiles (**Figure 4.12**). This image was compared to a dendrogram derived from the allelic profiles of the 49 Italian isolates characterised by MLST (**Figure 4.13**) which was produced using 'web tools' on the PubMLST database. No obvious relationships could be identified between isolates which were grouped together on the tree and the phenotypic and epidemiological data available for these isolates. There was also limited correlation between the two trees, which, despite the fact that they were derived using different algorithms and

Table 4.2: Cluster assignment, based upon mass profiles of isolates with the same multi-locus ST.

Isolate	Source	ST	Cluster
I2	Manca, VT (Ovine)	293	9
I8	Manca, VT (Ovine)	293	9
I10	Cricchi Valerio, RM (Ovine)	299	3
I11	Cricchi Valerio, RM (Ovine)	299	3
I17	Marini A. M., VT	304	3
I26	Greci, RM	304	3
I20	Fabi, FR	307	4
I48	Coculo, RM	307	2
I25	D'Angelo, RM	312	4
I46	D'Angelo, RM	312	2
I30	Paniccia, RM	316	4
I31	Paniccia, RM	316	4
I35	Colognesi, RM	318	2
I38	Colognesi, RM	318	2
I45	Maggi, RM	318	4
I18	Coculo, RM	305	1
I37	Cinque Stelle, RM	305	3
I36	Colognesi, RM	305	2
I40	Colognesi, RM	305	2
I47	Colognesi, RM	305	2
I41	Cremona, RM	319	3
I42	Cremona, RM	319	8
I43	Cremona, RM	319	8

There was a trend for isolates belonging to the same ST and/or originating from the same farm, to be placed within the same cluster based on similarities in mass spectra.

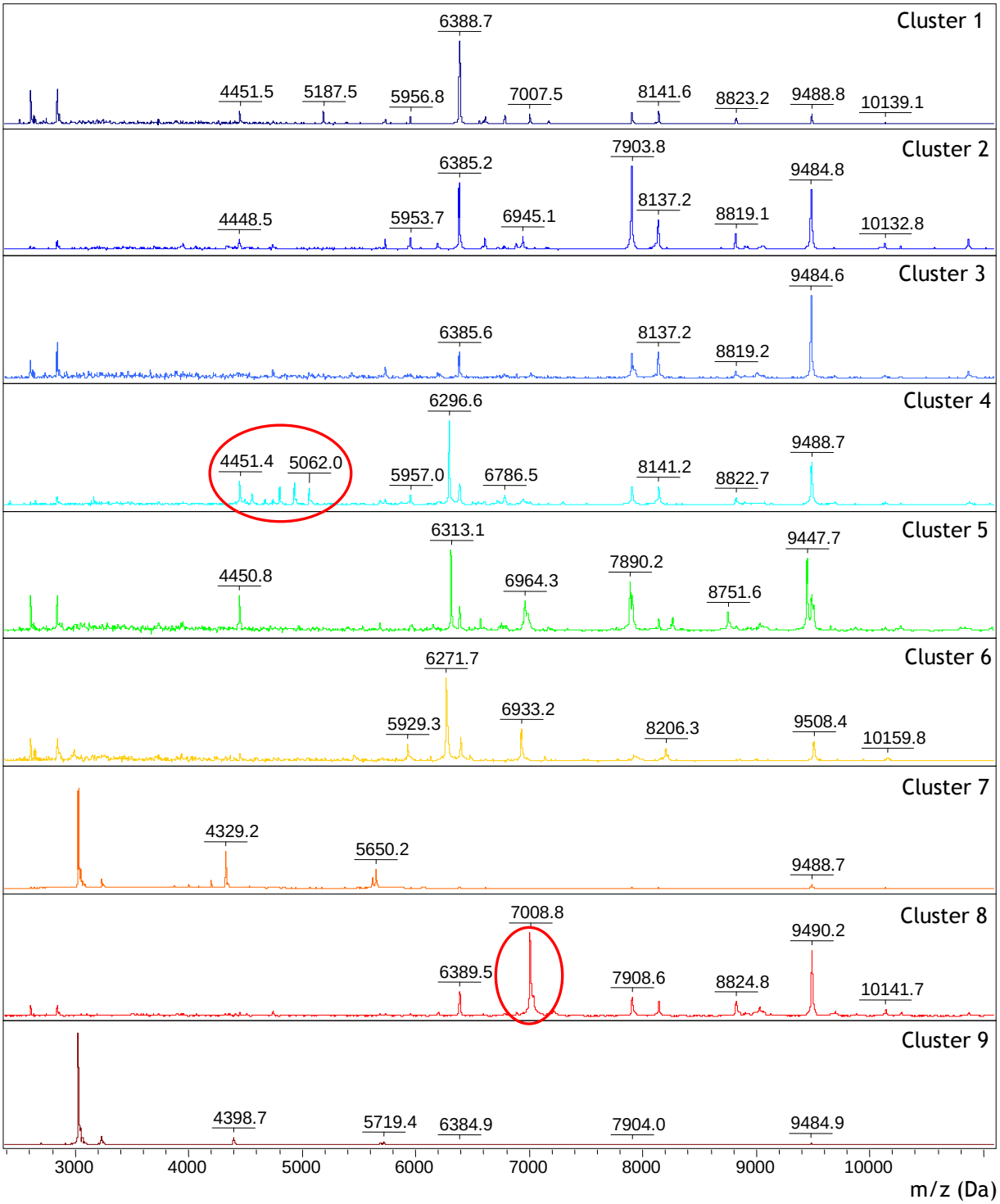


Figure 4.11: Mass profiles representative of each of the nine clusters identified from BioTyping of Italian *S. uberis* isolates. Profiles representing clusters 1, 2, 3, 5 and 6 were similar, whilst those for clusters 7 and 9 are quite poor. Very distinctive peaks on profiles representing clusters 4 and 8 are circled in red.

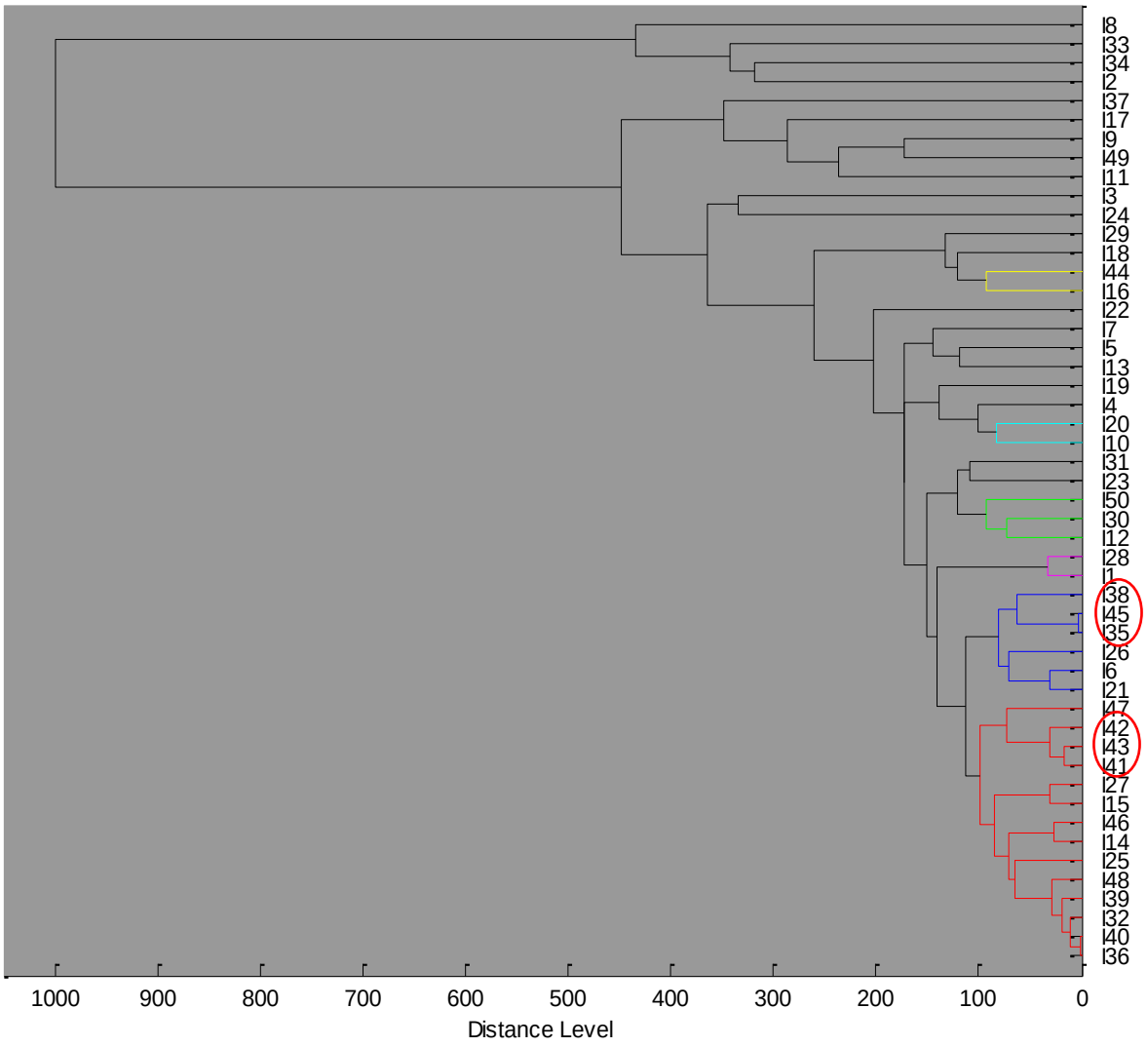


Figure 4.12: Score oriented dendrogram produced using BioTyper software to demonstrate the relationships between main spectra of Italian *S. uberis* mastitis isolates. Default settings for pre-processing spectra for MSP were used; including peaks within the mass range of 4,000 and 9,500 Da with a resolution of 1 Da. The maximum number of peaks to be included in pre-processing was 25, and these were required to have a threshold value of at least 0.001. Default settings were used for both MSP projection and dendrogram generation. Isolate groupings which correlated with the groupings identified from isolate allelic profiles (**Figure 4.13**) are circled in red.

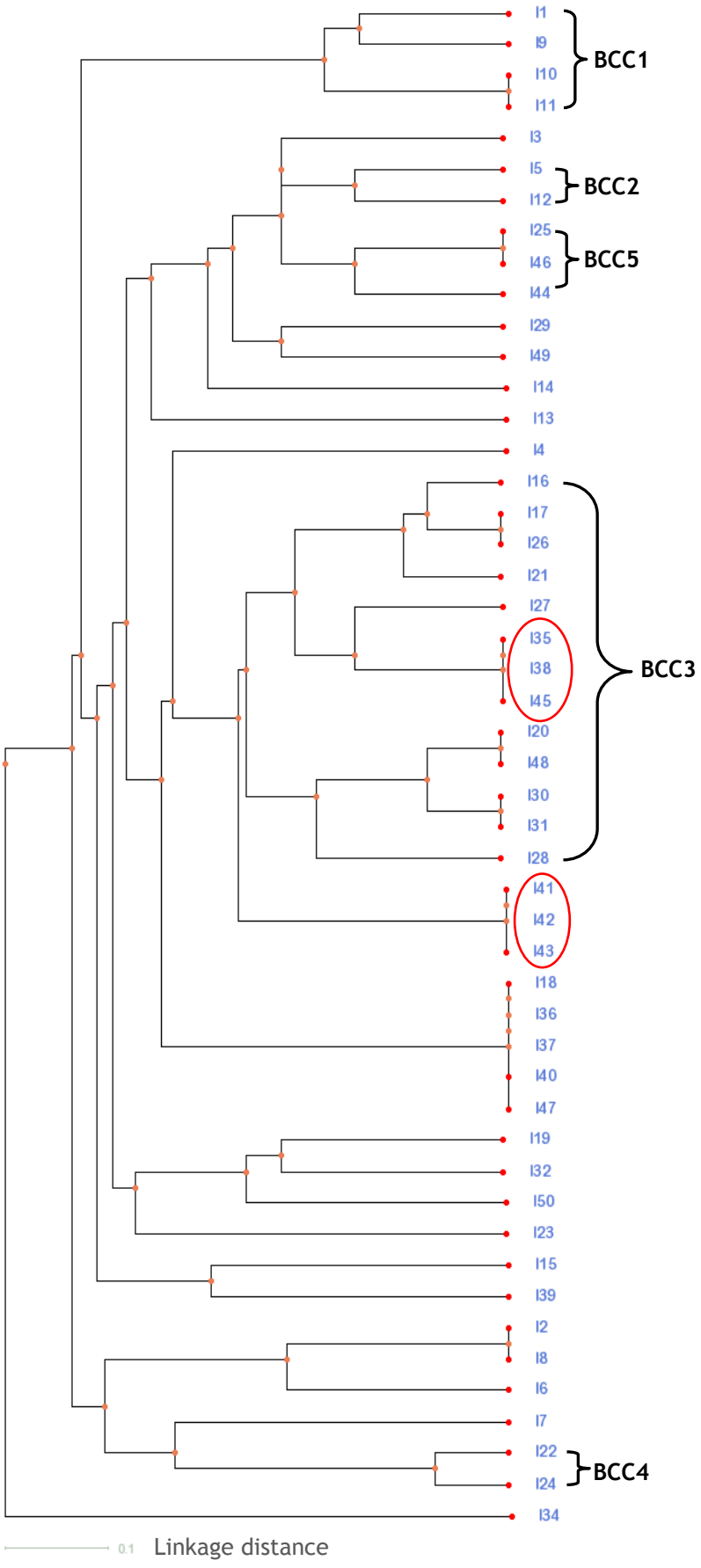


Figure 4.13: Dendrogram derived from MLST allelic profiles of Italian *S. uberis* isolates. Tree was generated using the UPGMA clustering algorithm and using the EMBOSS suite of programmes which derive relationships from allelic profile data by linking to the PubMLST website. The rendered Newick file was transferred to PHY-FI tree drawing tool (Fredslund, 2006). Isolates belonging to BURST groups (BCC) 1 to 5 are identified to the right of the tree, and where isolate groupings correlated to the relationships derived from mass profiles (**Figure 4.12**), these are circled in red.

programmes, suggests there is little correlation between the relationships inferred by MLST and MS. Isolates I41, I42 and I43 (ST 319), were grouped together on both figures, suggesting that the implied genetic homology of these isolates may indeed correlate with phenotypic homology. Similarly, I35, I38 and I45 (ST 318) were grouped together on both trees. Neither tools added bootstrapping support to the trees so it was difficult to draw any further conclusions from these results.

Limited relationships between the clustering obtained by BioTyping and the origin of the Italian isolates as well as the multi-locus ST could be identified. It appears that as the protocol stands, mass profiles of most isolates are generally too similar to allow precise differentiation. Further optimisation of the protocol and improved knowledge of the software might improve the clustering achievable, and identify additional links between datasets. Additional analysis of the UK isolates, where greater information is available regarding virulence, would be useful to judge the relevance of the clusters derived by BioTyping, but could not be completed during the time-frame of this study.

4.3 Discussion

The utilisation of MALDI-ToF MS as a simple, cheap and rapid alternative method for identification and typing of microorganisms is becoming more widespread. The ability to discriminate MRSA from MSSA isolates, and to correlate results with those generated by the considerably more time consuming protocols like PFGE (Edwards-Jones *et al.*, 2000; Walker *et al.*, 2002), exemplify the potential of this protocol. The main aims of the research described in this chapter were to develop a whole cell MS protocol for the characterisation of *S. uberis* mastitis isolates and to explore the extent of the discrimination that can be achieved. If successful, the technique may offer an alternative to more complex, expensive, genomic typing methods such as MLST, for characterising mastitis isolates.

The bacterial proteins visualised on mass profiles will be those which have been exposed during sample preparation, and thus are free to be ionised during MS. Previous research has demonstrated that addition of a simple step to disrupt the thick peptidoglycan cell wall of Gram-positive bacteria, noticeably improves the quality of the mass spectra produced (Smole *et al.*, 2002; Vargha *et al.*, 2006; Williams *et al.*, 2003). It was therefore not unexpected, that spectra produced from *S. uberis* 0140J cells scraped directly from a colony on an agar plate did not yield any peaks. Profiles obtained following the inclusion of an additional step to prepare cells were varied. Suspension in ethanol failed to produce suitable spectra, and may be attributed to the fact that ethanol has not always been found to sufficiently lyse cells (Madonna *et al.*, 2000), especially those belonging to Gram-positive genera, meaning that limited proteins are exposed during MS. Mass spectra produced following incubation of cells with lysozyme were dominated by two intense peaks, both of which were attributable to ions derived from the lysozyme itself, and which appear to suppress all additional peaks, as has been observed previously (Smole *et al.*, 2002). Alternatively, ribolysing or suspending cells in acetonitrile proved to be efficient and reproducible techniques for preparing *S. uberis* cells for MS, as a number of distinct peaks were consistently identified. Profiles produced using the two different protocols were visually distinct, with more peaks, covering a slightly broader mass range being identified following ribolysis. This was unsurprising, given that ribolysis completely disrupts the cell wall, releasing intracellular components, whilst acetonitrile is generally considered more to characterise cell wall associated components, although acetonitrile has also been shown to permit the release of intracellular proteins from bacteria of several distinct genera (Haag *et al.*, 1998; Mandrell *et al.*, 2005; Welham *et al.*, 1998). This may explain why,

in this study, a considerable number of peaks with identical mass were later found to be conserved on the profiles obtained from *S. uberis* 0140J cells using both protocols. These conserved peaks are likely to represent ribosomal proteins as these proteins have been frequently identified in previous studies from MS profiles, even when cells are solubilised directly in acetonitrile (Ryzhov and Fenselau, 2001). It seems therefore, that, in most cases, cells are in fact lysed by acetonitrile, permitting the detection of high abundance intracellular proteins.

During pilot studies, ribolysing or solubilising cells in acetonitrile prior to MS permitted clear discrimination between nine distinct *S. uberis* strains. Interestingly, clustering of mass spectra obtained from acetonitrile treated cells identified a relationship between strains belonging to the ST 5 CC, as determined by MLST conducted in **Chapter 3**. Two previous studies were found in which MS results also correlated directly with genomic typing methods; these showing that well described epidemic MRSA strains, indistinguishable by conventional typing methods, also had identical ICMS profiles (Bernardo *et al.*, 2002; Edwards-Jones *et al.*, 2000). Additionally, identical mass patterns were visualised from isolates obtained from different patients on a hospital ward during a disease outbreak, these being notably distinct from epidemiologically unrelated isolates; observations of clonality or heterogeneity made using MS were subsequently confirmed by PFGE (Bernardo *et al.*, 2002). The results presented here also demonstrated, however, that relationships exist between *S. uberis* strains from different countries that are unrelated by ST, suggesting that strains with different multi-locus STs may in fact display the same phenotype. Considering that MLST only targets several genes from the whole chromosomal complement for analysis, these results are thus not entirely surprising (as discussed later).

Whilst larger-scale ribolysis of cells becomes a relatively labour-intensive task, limiting the rapidity of the protocol, in contrast, preparing cells using acetonitrile has the potential for extremely high throughout. During analysis of mastitis isolates, it was found however, that 26 % were highly encapsulated, and could only be weakly solubilised directly in acetonitrile. Resulting mass spectra from these isolates were thus poor, and were dominated by a single high intensity peak, which may be attributed to the ionisation of capsular material. Interference of hyaluronic acid capsule in proteomic studies of *Streptococcus zooepidemicus* has similarly been described, and the authors used hyaluronidase followed by an extensive combination of chemical and physical lysis to prepare proteins for two-dimensional electrophoresis (Marcellin *et al.*, 2009). Interference can be attributed to the capsule increasing cell buoyancy and impeding

cell pellet formation during centrifugation (Marcellin *et al.*, 2009) as well as providing protection against cell lysis (Encheva *et al.*, 2006), explaining the limited solubility of capsular isolates in acetonitrile during this study. Hyaluronidase cleaves the bonds between *N*-acetylglucosamine and D-glucuronic acid subunits, catalysing the hydrolysis of the hyaluronic acid capsule which lowers the viscosity and increases the capsule permeability. Hyaluronidase was also used in this study to degrade capsule prior to re-suspension of cells in acetonitrile. This permitted the generation of high quality mass spectra from a mucoid strain, without affecting the profile obtained from a non-mucoid strain. Incubation of cells for 2 h at 37 ^{o}C in hyaluronidase subsequently permitted mass profiles to be obtained for all 50 Italian isolates analysed. Hyaluronidase treatment considerably extended the protocol however, and the additional processing appeared to lower the reproducibility of the mass spectra amongst replicate samples from the same isolate, such that subsequent clustering of different isolates was poor. Visual comparison of mass peaks in a pseudo-gel view demonstrated, however, that specific peaks (presumably corresponding to cellular proteins) were present or absent in sub-sets of isolates with the same ST, CC or those originating from the same farm. This demonstrates the potential of MS for the characterisation of *S. uberis* isolates. Additional analysis would be required however, to further support this observation and determine the identity of these proteins and whether their presence or absence in individual sub-sets was significant and related to virulence.

Despite appearing to be a suitable, more rapid, alternative solution for preparing mucoid *S. uberis* isolates for MS, ribolysis to physically disrupt cells, followed by solubilisation in acetonitrile, also did not consistently produce acceptable profiles from all replicate samples. Instead 10.5 % of the total spectra were discarded to avoid interference with subsequent clustering analysis. Unsurprisingly, poor spectra were mostly derived from isolates, which in previous experiments also could not be solubilised directly in acetonitrile (suggesting excessive capsule production). This implies that ribolysis is also unable to completely lyse cells in the presence of excess capsule. Clearly, a combination of hyaluronidase or chemical treatment prior to physical lysis is required to effectively deal with these cells; perhaps growth on agar plates containing hyaluronidase followed by ribolysis and finally re-suspension in acetonitrile would be effective. This type of extended lysis regime has been shown to be effective in proteomic studies with encapsulated Gram-positive bacteria (Marcellin *et al.*, 2009; Nandakumar *et al.*, 2000). The increased processing time that would be incurred using this method would thus appear to be a necessary addition to this protocol, and as such the improvement of subsequent large-scale reproducibility is a clear priority.

Comparison of the remaining mass spectra from Italian *S. uberis* isolates, using PCA and clustering software incorporated in the BioTyper programme, permitted assignment of spectra into nine clusters. In most cases, replicates from the same isolate were assigned to more than one closely related cluster, although one cluster generally contained most of the replicates from each isolate. All replicates from 16 isolates were however, all placed within the same cluster suggesting spectra obtained from these isolates were highly reproducible. Notably, one cluster comprised 9 isolates, and these, and all replicate spectra, were particularly well resolved. In the spectra from this cluster, four distinct, unique peaks were identified within the mass range of 4,450 to 5,050 Da. These common features, which should represent proteins, are shared among isolates which are not related by ST or origin. The identity of these features has not, at this point been determined, but may prove to be of interest for future research.

Clustering also demonstrated, as in an earlier pilot study, that some isolates representing the same ST also produced highly similar mass profiles. Most interestingly, within two sets of isolates sharing the same ST, those isolates which were also from the same farm were placed into the same cluster, whilst those from a different farm were clustered separately; this suggests that in some cases BioTyping may have the potential to provide additional discrimination over MLST. The main spectra of isolates were compared and visualised by dendrogram generation; these relationships were then compared to those inferred between isolates based upon similarity in their allelic profiles. No obvious relationship between isolates grouped by mass profiles could be seen, however, and even isolates placed by the software within the same clusters did not group together on the dendrogram. Only marginal correlation between the two figures was seen, with just two groups of 3 isolates (representing the same ST) being similarly grouped on both trees. Due to the limitations of the software, the two trees were derived using different algorithms and a lack of bootstrapping prevented conclusive statements from being made as to the confidence of the suggested groupings.

The following observations may explain the lack of congruence between the BioTyper and MLST-derived trees. Firstly, since MLST is targeted towards analysis of nucleic acid sequence, this technique identifies and differentiates between isolates with as little as a single change in the nucleotide sequence of target genes; however, these changes may not affect the translated protein sequence. Consequently, the same protein expressed from different alleles containing silent mutations would be indistinguishable by BioTyping. Considering that just 1 quarter of all the nucleotide polymorphisms

identified by MLST in **Chapter 3** resulted in an amino acid change, a considerable amount of DNA variation will not be detected by MS.

Secondly, genetic typing of seven housekeeping genes, as forms the basis of MLST, represents less than 0.5 % of the entire coding sequence of the *S. uberis* genome; thus, homology between the housekeeping genes of two isolates does not guarantee that the genomes are identical. Indeed, *S. uberis* isolates with the same ST have been found to differ in their PFGE profiles (Rato *et al.*, 2008; Tomita *et al.*, 2008) and in their RAPD profiles (**Chapter 3**). Genes encoding exposed or secreted proteins are instead more likely to be involved in virulence and thus variation of these sequences may relate more closely to pathogenicity. Furthermore, it has been demonstrated that bacterial virulence can be widely influenced by mobile genetic elements and the contribution of acquired virulence factors and antibiotic resistance genes to the pathogenicity of *S. aureus*, has been particularly well described (Sivaraman *et al.*, 2008; Goerke *et al.*, 2006; Lindsay and Holden, 2004). Additionally, protein profiles are further influenced by the regulation and expression of genes (Ziebandt *et al.*, 2010) which cannot be determined by MLST. Therefore, isolates with homologous housekeeping gene sequences do not necessarily share the same phenotype. Conversely, MALDI-ToF MS of bacterial cells has the potential to analyse any proteins from the cell, including those involved in virulence (even when these are derived from mobile genetic elements), and indeed differentiation between pathogenic and non-pathogenic bacteria by ICMS has been reported (Krishnamurthy *et al.*, 1996). Cold and acid tolerance proteins, as well as DNA and metal binding proteins, have also been identified from mass profiles (Arnold *et al.*, 1999; Holland *et al.*, 1999; Ilina *et al.*, 2010; Ryzhov and Fenselau, 2001) and these proteins may permit relevant discrimination between isolates corresponding to pathogenicity. In practice, however, highly abundant ribosomal proteins generally account for around half of the peaks on whole cell mass profiles (Arnold *et al.*, 1999; Ryzhov and Fenselau, 2001) and typically only approx. 20 to 40 peaks are visualised (Fenselau and Demirev, 2001). Characterisation based on MALDI spectra thus in fact similarly accounts for only a very small part of the entire cell complement, with one study estimating that only approx. 2 % of *E. coli* proteins were represented on mass spectra of whole cells (Ryzhov and Fenselau, 2001). Differentiation based upon just 20 abundant peaks from thousands of potentially expressed proteins in the cell, may thus also be limited, especially considering that the vast majority of proteins within strains of the same species will be similar (Dai *et al.*, 1999).

The number of peaks derived from ICMS which are informative for strain variation (and thus potentially virulence indicators) may therefore be limited. Of three studies analysing different strains of *H. pylori*, two of these identified only 5 or 6 peaks (approx. half of the total peaks reproducibly identified in each strain) as being strain specific (Ilina *et al.*, 2010; Winkler *et al.*, 1999). In the third study, more than 50 peaks were identified from a collection of 6 strains and a third of these peaks were unique to a single strain (Nilsson, 1999) suggesting greater potential for discrimination between strains. Chromatographic fractionation of the supernatant from solvent solubilised *E. coli* cells prior to MS, identified vastly more peaks than direct MS, demonstrating that ion suppression significantly affects mass spectra (Dai *et al.*, 1999) and identifying a method for increasing the number of strain specific informative sites available. Therefore, whilst whole cell MS does not intentionally target a particular set of proteins, only those which are the most abundant and easily ionised will be rapidly detected without fractionation. These limitations may indeed prevent the BioTyping technique from accurately differentiating between closely related isolates despite being proven to permit accurate and reproducible species differentiation to a level comparable to gene sequencing (Mellmann *et al.*, 2008). In this study, as has been shown previously (Ilina *et al.*, 2010; Winkler *et al.*, 1999), variation between strains was apparent, however, the reproducibility of replicates from an individual isolate hindered the differentiation between isolates with similar peak profiles.

A lack of additional phenotypic or epidemiological information regarding the Italian isolates prevented any biological relevance being attributed to the clusters identified or the relationships inferred by the dendrogram created using the BioTyper analysis software, which may more conclusively demonstrate or disprove the usefulness of this technique. Instead, in this study, apart from limited exceptions, no significant association could be made between isolate genotype, origin, and the clustering visualised based upon mass profile similarities. This was in agreement with a previous study typing clinical *Helicobacter pylori* strains (Ilina *et al.*, 2010). Time restraints similarly prevented whole cell MS of *S. uberis* mastitis isolates from the UK collection from being completed, and thus determination of any correlation between mass profiles and persistence or non-persistence. Identification of proteins responsible for peaks which were conserved or absent from a sub-set of isolates was also not determined in this study, and may represent an additional future research avenue.

The use of whole cell MS as an alternative typing method is attractive as it is quick, cheap and offers high throughput. The estimated full economic cost of analysing 1

isolate using MS is under £14, including overheads and staff costs, and for a collection of 48 strains requires 11 people hours (Prof. D. G. E. Smith, personal communication). To complete MS, approx. 30 min is required per isolate to prepare the cells for MS. In comparison, sequencing of 7 gene regions, in both directions, as is required for MLST, costs between £20 and £90, depending upon the company used, and in addition, expensive reagents are required for DNA extraction and PCR. The total time required to complete the MLST process and edit sequence data is in the region of 4 hours for 1 isolate, although this is reduced when processing multiple samples in parallel. BioTyping may thus offer a cheaper and quicker alternative to MLST. In this study, it has been demonstrated however, that additional cell processing is required to remove the hyaluronic acid capsule produced by many *S. uberis* isolates, before suitable mass profiles can be obtained. This processing stage, remains undoubtedly quicker and easier, however, than the processing and sequencing required for genomic typing. The use of MS for distinction between strains of the same species, unlike species identification, is still in its infancy. In this study, small-scale analysis of limited numbers of strains produced highly reproducible mass profiles between replicates and during distinct experiments. Reproducibility achieved during scaled-up experiments was however, notably poorer, and requires further optimisation. A standardised protocol utilising formic acid, has since been described by the manufacturers of the BioTyping software, which they claim is suitable for preparing diverse species for MS (Maier *et al.*, 2008). In a recent study, Gram-positive bacteria were correctly identified more frequently following preparation with formic acid than when just acetonitrile was used, but this remained notably lower than the identification accuracy for Gram-negative species (La Scola and Raoult, 2009), suggesting further improvements are possible. The use of formic acid may also permit direct lysis of cells with excessive capsule, since it has previously been used to successfully lyse rigid, polysaccharide-rich fungal cells (Amiri-Eliasi and Fenselau, 2001). This standardised protocol could help to overcome the reproducibility problems encountered in this study.

Despite clear limitations, this study has demonstrated the promise of MS as an alternative typing tool for *S. uberis* mastitis isolates. Discrimination between isolates was achieved, but a limited correlation to their origin or ST was seen. This is the first application of BioTyping using MALDI-ToF MS to the characterisation of mastitis pathogens, and demonstrates the possibilities offered by the protocol. Although not pursued during this project, further application of the protocol for biomarker assignment for rapid identification of field isolates, informing treatment decisions, or for identification of protein peaks conserved amongst individual populations which may

identify novel vaccine candidates are feasible. Furthermore, a successful, rapid typing protocol could equally be utilised for the typing of additional mastitis pathogens.

Chapter 5: Phenotypic analysis of *S. uberis*

5.1 Introduction

In order to establish an infection, bacteria must acquire specific nutrients to allow them to proliferate; these requirements being dependent upon the species in question and the niche to be inhabited. Bacteria thus have evolved the ability to sense their extracellular environments and adapt accordingly. Bacterial virulence is therefore also highly regulated by environmental stimuli, since environment-induced changes in gene expression can generate altered cellular phenotypes, better suited to survival within that environment (Mekalanos, 1992). Consequently, the use of a chemically-defined medium (CDM) is a perfect means for studying how an organism may behave *in vivo*. Defined media have been widely utilised to explore the effects of environmental conditions on bacterial growth, and on the expression of different factors. The effects of magnesium starvation on membrane permeability and subsequent viability of *E. coli* cells has, for example, been demonstrated (Fiil and Branton, 1969), while zinc has been shown to stimulate the optimum production of a virulence determinant by *Burkholderia pseudomallei* (Percheron *et al.*, 1995). More recently, regulation of virulence genes, enzymes and transporters has been shown to be linked to the nutritional status of *S. pyogenes* cells (Malke *et al.*, 2006; Shelburne, III *et al.*, 2008). A CDM has also been used to determine the optimum growth conditions for the production of pertussis toxin to improve vaccine production (Thalen *et al.*, 2006). Chemically-defined media have been described for a number of pathogenic bacterial species; in the case of *S. aureus*, for example, CDM permitted a growth defect in a mutant strain to be observed (Doherty *et al.*, 2006), whilst a CDM for Group A streptococci was optimised which supported growth of 20 strains with kinetics comparable to growth in complex medium (van de Rijn and Kessler, 1980). A CDM suitable for culturing *S. uberis* has also been described, and addition of casein peptides to this medium was shown to improve resistance of cells to killing by neutrophils, this resistance not being influenced by capsule production (Leigh and Field, 1991). These published media shared a complex composition, requiring the individual addition of between 30 and 50 different components.

The pathogenesis of *S. uberis* remains poorly understood. Several factors which may be related to virulence have been identified, and these have been discussed previously in **Chapter 1**. It seems, however, that no individual factor is responsible for bacterial persistence and hence the induction of mastitis. It has been suggested that to survive in the mammary gland *S. uberis* must have the ability to avoid detection and destruction by host defences as well as the ability to proliferate in milk (Smith *et al.*, 2003). Potential methods by which *S. uberis* acquires nutrients have been discussed previously

in this thesis. There is, however, no full understanding of the mechanisms by which this organism colonises and persists within the mammary gland. Consequently, the availability of a suitable CDM could allow this area of research to be addressed. There are additional host factors which would be particularly relevant to assess using such a medium, which have not (to the author's knowledge) been the focus of any previous reports involving *S. uberis*; however, their relevance for other bacteria makes them valid targets of study within the context of *S. uberis*. These factors are discussed in further detail in the following paragraphs.

Iron is generally recognised as an element which is essential for bacterial survival as it is required for electron transport, amino acid biosynthesis and DNA synthesis, as well as for several additional important functions. Only a few exceptional species have been described, such as lactobacilli (Elli *et al.*, 2000) which are able to proliferate in the absence of iron. Iron availability is thus tightly regulated in the host by cytokines, which act on the liver, causing the release of lactoferrin and transferrin to bind and limit free-iron as part of the acute phase response to infection. Such a reduction in iron has been demonstrated in the serum of cattle during mastitis infections with *E. coli* (Erskine and Bartlett, 1993) and *S. aureus* (Middleton *et al.*, 2004); thus, for bacteria to persist and cause mastitis, mechanisms must exist by which they can compete with host proteins to acquire limited metal ions.

Proteins produced by *S. uberis* have been described which have the ability to bind lactoferrin. These proteins, Lbp (Moshynskyy *et al.*, 2003) and SUAM (Almeida *et al.*, 2006) may be secreted in an attempt to sequester iron from host iron-binding molecules. Mutants lacking Lbp have been shown however to survive in iron deficient medium (Moshynskyy *et al.*, 2003) suggesting an alternative pathway for bacterial iron acquisition exists. These lactoferrin-binding proteins may instead contribute to the adherence of bacterial cells to bovine mammary epithelial cells (as discussed previously in **Chapter 1**).

A mechanism commonly used by pathogenic microorganisms for iron acquisition involves the secretion of low molecular weight chelators called siderophores which have such high affinity for iron that they can liberate iron bound to host proteins. Iron-siderophore complexes are then transported across the bacterial membrane for use in the cell. Two types of siderophore have been identified; the catechols and the hydroxamates, which are structurally distinct and therefore differ in their methods for binding iron. Many bacteria, including *S. agalactiae* (Clancy *et al.*, 2006), *S. aureus* (Courcol *et al.*, 1997;

Lim *et al.*, 1998; Lindsay and Riley, 1994; Park *et al.*, 2005) and *E. coli* (Dall' Agnol and Martinez, 1999) have been shown to utilise siderophores for the liberation of bound iron from host sources such as lactoferrin and transferrin. To date, no such siderophore production by *S. uberis* has been demonstrated. The importance of the siderophore system in the pathogenesis of *S. aureus* has, however, been highlighted using a mouse infection model, where a mutant that failed to produce siderophore was unable to persist and cause infection (Dale *et al.*, 2004a).

Siderophore production in *S. aureus* has thus been well studied, and the genes involved in siderophore production described in great detail (Dale *et al.*, 2004a; Dale *et al.*, 2004b). In *S. aureus,* siderophore-mediated iron uptake involves complicated systems, including a nine-gene iron regulated operon, *sbn* which produces the siderophore staphylobactin (Dale *et al.*, 2004a), and the *sirABC* operon which is involved in the transport of iron-staphylobactin complexes into the cell (Dale *et al.*, 2004b). Whilst iron is essential for bacterial growth, it is also highly toxic when over-accumulated. Intracellular iron concentrations must therefore be well regulated, and in bacteria, including *S. aureus,* this is typically conducted through ferric uptake regulatory (*fur*) systems (Horsburgh *et al.*, 2001; Xiong *et al.*, 2000). Binding of Fur to the *sir* operon promoter in *S. aureus* has been shown to regulate siderophore expression and control iron uptake (Xiong *et al.*, 2000).

In *S. aureus,* iron restriction promotes the expression of additional proteins as well as siderophores, these proteins being well conserved amongst different isolates (Courcol *et al.*, 1997). Iron limitation in *Corynebacterium diphtheriae* similarly triggers the expression of additional virulence factors, including adhesins and toxin, which improve survival, but which are not required for iron uptake (Moreira *et al.*, 2003). Iron limitation may thus increase the pathogenicity of bacteria, as numerous virulence factors are expressed which directly and indirectly facilitate survival in the host. Iron starvation can thus also be utilised as a method for identifying additional bacterial virulence factors which may function as potential vaccine candidates.

An additional bacterial survival mechanism which can be induced under certain growth conditions, such as iron limitation, is biofilm formation (Arrizubieta *et al.*, 2004; Johnson *et al.*, 2005). The contribution of bacterial biofilm formation to virulence through the promotion of survival in the host has been increasingly studied in recent years. The formation of bacterial biofilms is mediated by the production of slime, or bacterial exopolysaccharide matrices, which are loosely bound to the cell wall of slime

producing (SP) bacteria, as demonstrated by electron microscopy, immuno-fluorescence and ease of recovery in culture supernatants (Baselga *et al.*, 1993). The slime layer provides a degree of protection to bacteria encapsulated within it and also serves to collect nutrients from the environment, concentrating them for bacterial use (Costerton *et al.*, 1987). Additionally, the exopolysaccharide complex, or glycocalyx as it is also known (Costerton *et al.*, 1987), permits the attachment of bacteria to inert or biotic material and to each other, allowing the subsequent formation of micro-colonies or biofilms.

Biofilm formation has now been shown to incorporate distinct stages, with cells undergoing considerable phenotypic change between stages and differing considerably in their protein expression profiles compared to planktonic cells (Sauer *et al.*, 2002). Biofilm formation begins with primary or reversible adhesion of the bacteria to a surface which is generally mediated by electrostatic and hydrophobic interactions (Carpentier and Cerf, 1993). Where conditions favour the propagation of primary adhesion, and no external intervention occurs, additional molecules such as exopolysaccharide are utilised to bind the bacteria irreversibly to the surface. At this point planktonic organisms may complex with surface bound bacteria forming an aggregate. Modifications in gene transcription permit the switching of bacteria from planktonic to sessile forms under different environmental conditions which allows interaction between bacterial cells or a surface. *S. aureus and Staphylococcus epidermidis* for example, produce a polysaccharide intercellular adhesion (PIA) protein that has been shown to be important for cell to cell adhesion and biofilm formation, as disrupting the production of this adhesin impaired biofilm formation (Cramton *et al.*, 1999; Mack *et al.*, 1996). Finally, the biofilm matures as the density and complexity of the biofilm increases, dependent upon multiple factors, such as cell replication, death, availability of nutrients, removal of waste products, pH and oxygen perfusion. When the biofilm reaches a critical mass, the outermost cells are released and have the opportunity to disperse and colonise other surfaces. Interestingly, the protein patterns from these released cells were most similar to planktonic cells, with many of the identified biofilm associated proteins being down-regulated at this stage (Sauer *et al.*, 2002). Cell to cell signalling and density-dependent gene expression regulate the biofilm dynamics with communication between biofilm associated bacteria occurring via quorum sensing (QS) systems (McLean *et al.*, 1997). The importance of QS systems in the regulation of *S. epidermidis* and *S. aureus* biofilm formation have been demonstrated, and thus therapeutic applications for QS inhibitors identified (Balaban *et al.*, 2007;

Vuong *et al.*, 2003). The genetic basis for biofilm formation is a subject discussed at a later stage in this thesis.

Existence as a biofilm offers many advantages to the bacterial population. Close association of numerous bacterial cells permits the expression of unique combinations of virulence factors, which may promote disease. Due to nutrient limitation, bacterial cells in a biofilm generally have a lower metabolic rate and thus, particularly bacteria in the inner-most layers, are considered to be more resistant to antibiotic treatments than bacteria which remain planktonic. This protection has been illustrated *in vitro* in *S. aureus* biofilms (Amorena *et al.*, 1999; Melchior *et al.*, 2006a). Biofilm-associated bacteria are similarly better protected from host defences than planktonic cells, with slime producing *S. aureus* strains being shown *in vitro* to resist phagocytosis by bovine PMN (Barrio *et al.*, 2000) and to adhere better to ovine mammary epithelial cells, this adhesion being inhibited by antibodies against slime (Aguilar *et al.*, 2001).

Biofilm forming ability was found to be higher amongst *S. aureus* isolates from bovine milk than those from teat skin and milking machines, suggesting biofilm production is advantageous for survival in milk (Fox *et al.*, 2005). In animal infection models, *S. aureus* strains forming biofilms were shown to promote greater colonisation and cause more persistent infections but also to decrease bacterial loads, milk SCCs, and clinical signs in infected animals (Baselga *et al.*, 1994; Cucarella *et al.*, 2002; Cucarella *et al.*, 2004). Biofilm formation by *S. aureus* may thus render the bacteria less virulent, in terms of the severity of the mastitis infection, but this in turn permits the bacteria to remain undetected in the host. These observations have led to the widely accepted hypothesis that biofilm formation is important for the development of chronic mastitis conditions in which infection persists despite rigorous antimicrobial therapy (Aguilar *et al.*, 2001; Baselga *et al.*, 1994; Fox *et al.*, 2005; Melchior *et al.*, 2006a; Melchior *et al.*, 2006b; Vasudevan *et al.*, 2003).

As biofilm formation provides obvious benefits to *S. aureus* for survival in the mammary gland, it is not surprising that *S. epidermidis* and *E. faecalis* mastitis isolates have also been shown to form biofilms *in vitro* (Oliveira *et al.*, 2006; Toledo-Arana *et al.*, 2001). To date, no similar observations of biofilm production by *S. uberis* mastitis isolates have been published. Biofilm formation may however offer answers regarding the pathogenicity of *S. uberis* during mastitis infections, and potentially explain why isolates sensitive to antibiotics *in vitro* are resistant *in vivo*, and why some *S. uberis* infections persist while others are naturally cleared (Deluyker *et al.*, 2005; Milne *et al.*,

2005). The formation of biofilms may similarly explain why, in this study, isolates from both persistent and non-persistent mastitis cases shared identical STs, and why, according to another MLST study, isolates from short term and extended infections were similarly genetically identical at housekeeping gene loci (Pullinger *et al.*, 2007).

Multi-locus sequence typing conducted as part of this thesis could not identify unique genotypes responsible for either persistent or non-persistent infections (**Chapter 3**), but dominant strains which are presumably better adapted for survival in the mammary gland were identified in both the UK and Italian collections. The main aims of the work described in this chapter were to utilise defined media to determine how different elements affect the growth of *S. uberis in vitro* and stimulate virulence factors. This may offer an explanation for either the persistent phenotype or dominant genotype seen amongst *S. uberis* mastitis isolates analysed in this study. These factors may even prove to have potential as vaccine targets. A vaccine based upon exopolysaccharide derived from biofilm forming *S. aureus* has, for example, already been shown to reduce the frequency and severity of subsequent mastitis-causing *S. aureus* infections (Amorena *et al.*, 1994; Perez *et al.*, 2009). Similarly, siderophore receptor proteins have been successfully used to reduce mortality rate in chickens infected with *Salmonella enterica* (Kaneshige *et al.*, 2009), and reduce the prevalence of *E. coli* O157 in cattle (Fox *et al.*, 2009). To facilitate this objective, the first aim of this study was to produce a simple, yet reliable defined medium, generally representative of the *in vivo* milk environment, to which specific components could be added or removed; subsequently, the contribution of these components to *S. uberis* growth could be directly attributed.

5.2 Results

5.2.1 Development of a novel chemically-defined growth medium for *S. uberis*

In order to allow the *in vitro* study of *S. uberis* in an environment matching that which the organism would encounter *in vivo* as closely as possible, efforts were made to develop a CDM that would permit this objective. A CDM has previously been described which permitted *in vitro* growth of *S. uberis* (Leigh and Field, 1991); however, due to the complexity of this medium and observations of inconsistent growth (**Appendix 5**), a new CDM was developed. Commercial RPMI-1640 medium (Sigma) was utilised as the basal component of this medium, after previous success in our laboratory using this basic constituent for a defined medium which supported the growth of *Corynebacterium pseudotuberculosis* (Walker, 2009). This readily-available base was then further supplemented with a variety of components, and the ability of *S. uberis* to grow, and the extent to which it grew in each CDM formulation was assessed. Cultures of *S. uberis*, prepared from glycerol stocks and grown in BHI broth until stationary phase of growth (20 to 24 h) were diluted 100-fold and used to inoculate the defined media of interest. Growth kinetics were measured automatically using the Bioscreen C apparatus, and initial experiments were conducted using strain 0140J alone. Data derived from each stage of development of the final CDM formulation is not shown, however, it was found that RPMI supplemented with glucose and a combination of metal ions (**Section 2.3.1**) was able to support a higher level of *S. uberis* growth than the published CDM, and growth kinetics more closely mimicked those obtained in complex laboratory medium (**Figure 5.1**).

Growth of *S. uberis* in CDM was similarly achieved following inoculation directly from glycerol stocks which had been derived from cells cultured in BHI broth; however, passage of *S. uberis* cultures prepared in CDM into fresh CDM was not possible. Interestingly, supplementation of the medium with a final concentration of 0.5 % (w/v) hydrolysed casein restored growth of *S. uberis* 0140J following passage from CDM culture (**Figure 5.2**). Addition of hydrolysed casein to CDM inoculated with a culture prepared in BHI broth had no additional effect on growth (**Figure 5.2**). Similar growth observations were also seen when using the published CDM (data not shown). It was considered that using an *S. uberis* inoculum culture prepared in CDM with hydrolysed casein ensured that the bacteria did not have residual nutrient stores which permitted growth despite a clear lack of nutrients in the test medium. In this way it was

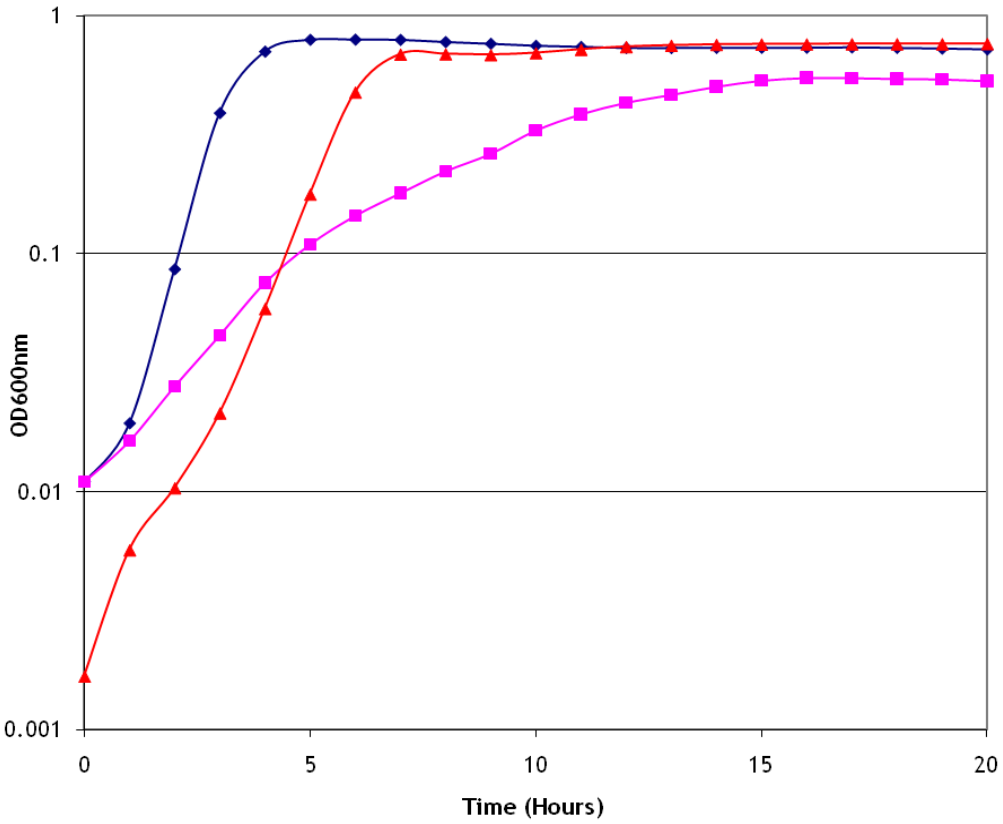


Figure 5.1: Growth characteristics of *S. uberis* 0140J in CDM and BHI broth. A stationary phase culture prepared in BHI broth was used to inoculate the test medium; BHI broth (◆), published CDM with glucose and metal ions (■) and RPMI CDM with glucose and metal ions (▲). Final growth levels achieved with RPMI CDM were similar to those in BHI broth despite a slower initial growth rate.

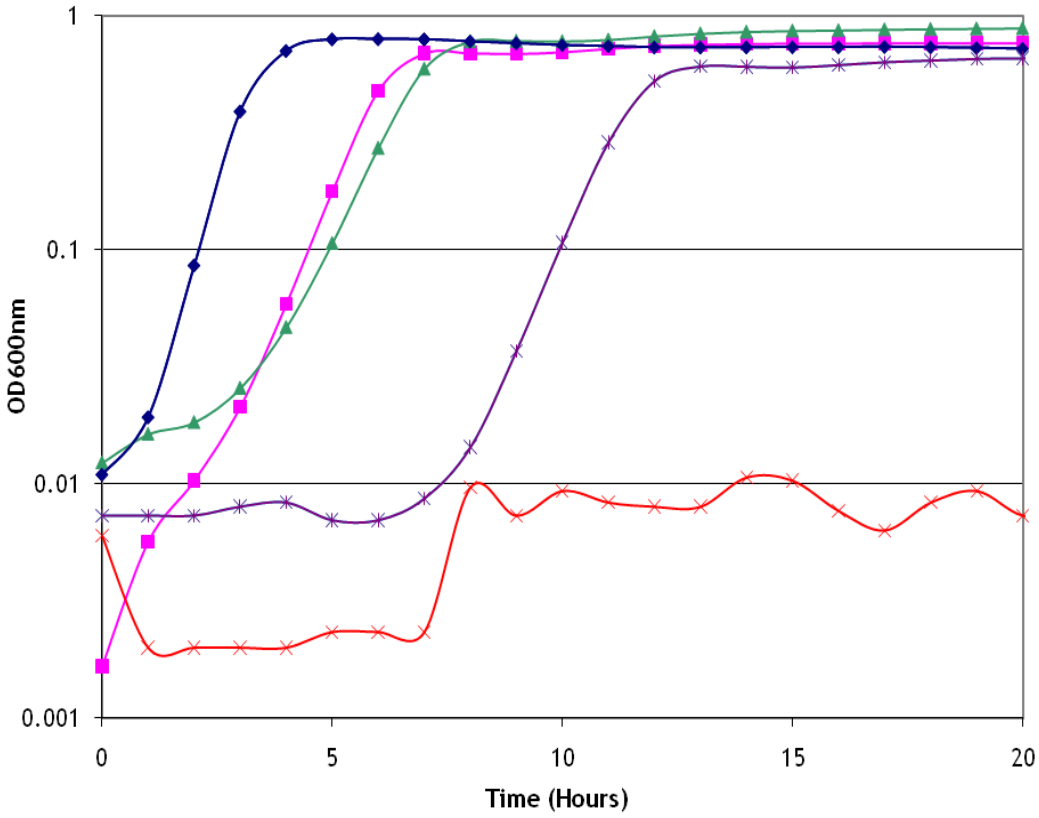


Figure 5.2: Inoculum dependent effect of hydrolysed casein on the growth of *S. uberis* 0140J in CDM. A stationary phase culture prepared in BHI broth was used to inoculate test medium; BHI broth ( ), CDM with metal ions ( ) and CDMch with metal ions ( ) whilst a CDMch culture was also used to inoculate CDM with metal ions ( ) and CDMch with metal ions ( ). Hydrolysed casein was clearly required to support growth when the inoculum was prepared in CDM, but when BHI broth was used to prepare the inoculum culture, addition of hydrolysed casein had no effect on subsequent growth.

considered that a more accurate identification of factors important to growth would be achieved.

To further attempt to recreate, *in vitro,* the *in vivo* environment of the bovine mammary gland, the carbohydrate component of the CDM was changed from glucose to lactose, since this is the most abundant sugar in milk. Growth rate and final growth levels achieved by *S. uberis* 0140J in lactose were good but slightly lower than in glucose and again hydrolysed casein was required to permit growth. Consequently, in subsequent experiments, lactose was routinely used in place of glucose. The inoculum used in this experiment had been cultured for just 4 h in defined medium prior to use as an inoculum, and thus was at the exponential phase of growth. As such, upon inoculation into medium of interest, the lag phase of growth was virtually eliminated, so stationary phase of growth was reached in approx. 10 h which corresponded roughly to the lag period previously required before the bacteria began to grow when using a stationary phase inoculum. Despite reducing the time required to complete the growth experiment, the mean generation time was lower (68 m) when a stationary phase inoculum was used, compared to when a mid-log phase inoculum was used (120 m). The maximum cell culture densities achieved using either inoculum were equivalent, but a major advantage of the adapted protocol (using a mid-log phase culture inoculum) was the fact that experiments could be completed in 24 h. The reproducibility of growth curves obtained on different days, using mid-log cultures to inoculate test medium was high and slightly greater than the reproducibility achieved when using stationary phase cultures (which was also high) to inoculate test medium (data not shown).

The requirement for hydrolysed casein to permit *S. uberis* growth *in vitro* was further demonstrated using a concentration gradient in CDM. As little as 0.1 % (w/v) hydrolysed casein (final concentration) supported growth, whilst 0.4 to 0.6 % (w/v) stimulated the highest growth levels, although limited differences were seen at these concentrations (data not shown). For subsequent growth studies, 0.5 % hydrolysed casein was used to prepare CDM (CDMch).

Growth patterns of further *S. uberis* strains were determined in CDMch. Fourteen of twenty *S. uberis* mastitis isolates produced very similar growth curves to that of *S. uberis* 0140J, reaching maximal $OD_{600\ nm}$ values of between 0.7 and 1.0 within 24 h. The remainder of the isolates grew more slowly and reached an $OD_{600\ nm}$ of at least 0.4 within the 24 h period (data not shown). As growth kinetics of all isolates in BHI broth was well conserved, this observation demonstrated that individual isolates differ in

their ability to utilise nutrients, implying that some isolates are less well (or better) adapted for survival in the mammary gland. These results also confirmed that the defined medium did not simply support growth of one individual strain, but was relevant for a diverse selection of clinical isolates.

5.2.2 Casein utilisation by *S. uberis*

To determine if *S. uberis* was able to directly utilise casein, this was added to the CDM (CDMcas) in place of hydrolysed casein to a final concentration of 0.5 % (w/v). Significantly, growth of the test strain (0140J) was still possible in this medium, despite exhibiting a significantly extended lag period. Interestingly, however, the maximal absorbance value (indicative of cell density) achieved was more than twice that achieved even in complex laboratory medium (**Figure 5.3**). Growth in CDMcas also appeared to be significantly enhanced in the presence of metal ions. As with hydrolysed casein, the growth rate and final cell density of *S. uberis* seemed to be proportional to the concentration of casein in the medium (data not shown). In the absence of casein or hydrolysed casein, growth of *S. uberis* could not be stimulated however, by the addition of an amino acids solution to the CDM.

Interestingly, flocculates were visible following growth of *S. uberis* in CDMcas which were not observed following growth in CDMch. Complete re-suspension of these particulates could not be achieved despite vigorous shaking. The effect of pH on the solubility of casein in CDM was therefore determined. The pH of a culture of *S. uberis* in CDMcas before and after growth was measured and found to be 7.1 and 4.0 respectively. Solutions of casein and hydrolysed casein in water were prepared, and the pH values adjusted to 7.0, 6.0, 5.0, 4.0 and 3.0 to cover the range of pH values observed during *S. uberis* culture. Whilst there was no change in the solubility of the hydrolysed casein solutions at different pH values, it was clearly visualised that a drop in pH below 6.0 caused the casein to precipitate out of solution, and at pH 4.0, casein clumps fell to the bottom of the tube (**Figure 5.4 B**). Western blotting was used to determine that casein is also degraded by this change in pH. When the casein became insoluble at pH values lower than 6.0, an anti-casein antibody (sheep polyclonal to casein by abcam - ab35189) could only identify a small, faint band from a mixture of the liquid and precipitated material obtained (**Figure 5.4 A**).

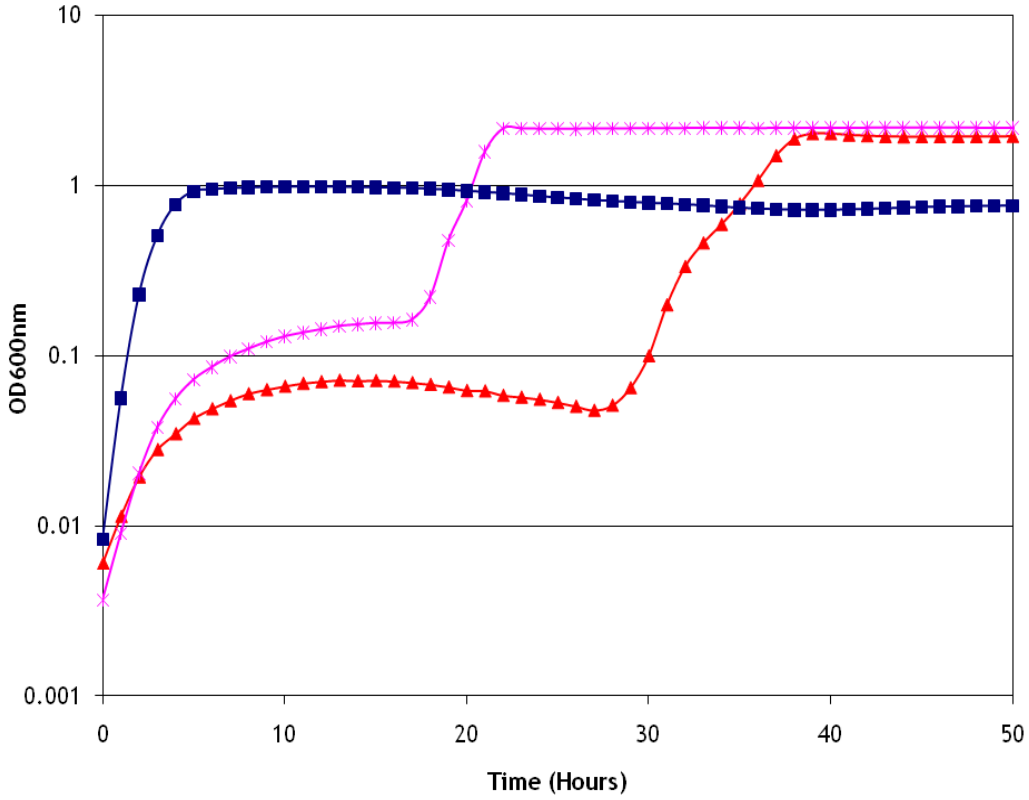


Figure 5.3: Growth of *S. uberis* 0140J in CDMcas and the effect of metal ions. A mid log phase culture was used to inoculate test medium; BHI broth (—■—), CDMcas (—▲—) and CDMcas with metal ions (—✳—). As can be seen, despite a significant lag phase, the final OD values following growth in CDMcas are much higher even than growth achieved in BHI broth. Addition of metal ions notably reduces the lag phase.

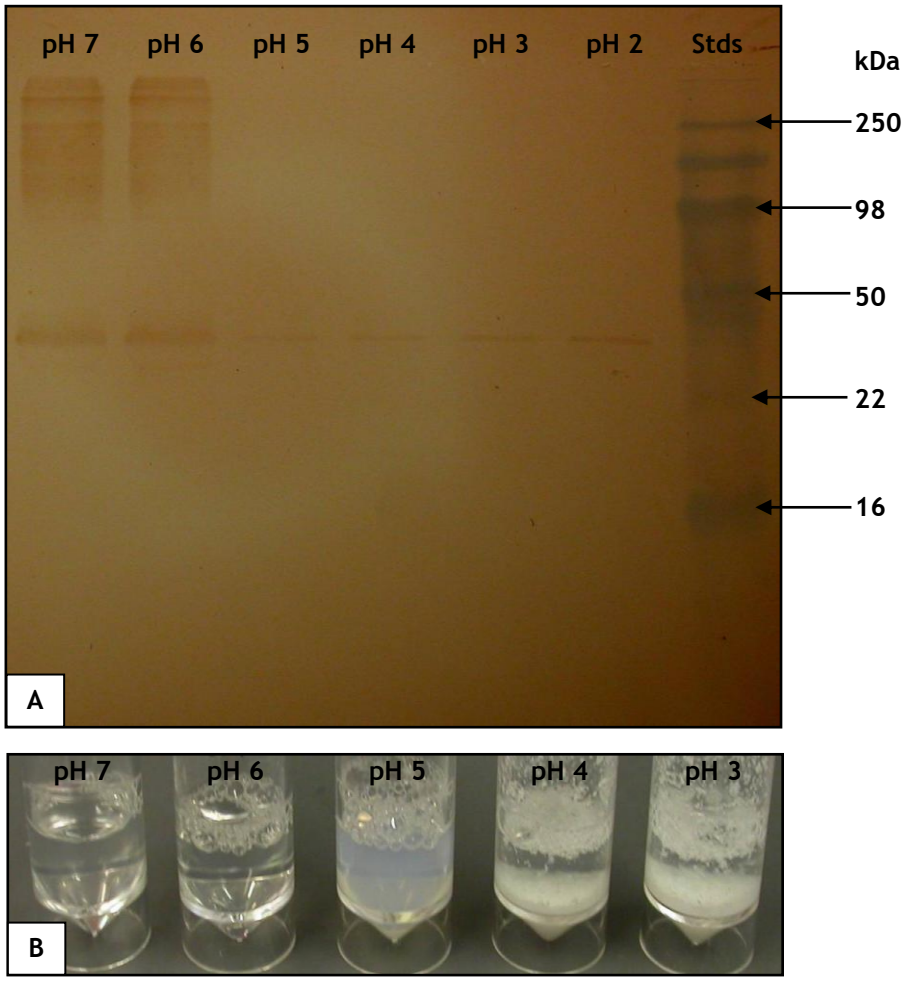


Figure 5.4: Effect of pH on the solubility and degradation of casein. At pH 6.0 and 7.0 western blotting, using an anti-casein antibody (ab35189) to detect casein, identifies large casein fragments as well as a smaller fragment, whilst below pH 6.0 only a the small fragment is detected and this band is notably fainter (A). In solutions of water adjusted to a pH of below 6.0, casein precipitation from solution is clearly visualised (B).

Growth of *S. uberis* 0140J in CDMcas was thus characterised further by measuring the pH, c.f.u/ml and $OD_{600\ nm}$ values during growth (**Table 5.1**). Growth of *S. uberis* accelerated at approx. pH 7.0 as determined by colony counts, with optimal growth seen at around pH 6.0. Growth subsequently decelerated once the pH dropped much below 6.0. Absorbance measurements at 600 nm contrastingly suggested steady growth until approx. pH 5.5 at which point there was a rapid increase in turbidity. In a cell-free, pH-matched control, at pH 5.5 the casein comes out of solution, explaining the rapid increase in absorbance value at this point. Measuring absorbance thus could not be utilised to accurately estimate the growth rate of *S. uberis* in CDMcas. It was apparent in the early stages of culture that OD did accurately reflect the true state of *S. uberis* growth; however, the inevitable drop in the pH of the culture caused the casein to become insoluble, leading to an artificial increase in OD. Colony counts also confirmed that *S. uberis* is able to grow in CDMcas before the pH affected the solubility of the casein.

The capacity of *S. uberis* to grow in CDM utilising casein directly, was further illustrated using a cell viability assay employing the alamarBlue® reagent (Invitrogen). AlamarBlue® uses resazurin, a blue, non-fluorescent, cell-permeable compound that becomes reduced upon entering viable cells to resorufin, a red highly fluorescent compound. As this conversion is continuous in viable cells, an increase in fluorescence, absorbance, or simply the visualisation of a colour change from blue to red, is an effective indicator of cell viability. AlamarBlue demonstrated that *S. uberis* cells are viable in CDMcas, and confirmed that viability was improved upon addition of metals ions to the medium (**Figure 5.5**). As expected, CDMch also supported viable growth, while CDM alone did not permit growth when a culture prepared in CDMch was used to inoculate the test medium. These observations supported the previous growth observations made using the bioscreen C apparatus.

5.2.3 *S. uberis* metal ion requirements

5.2.3.1 Growth of *S. uberis* under metal-ion-restricted conditions using chelators

The relative importance of different metal ions for the growth of *S. uberis* was initially explored by individually adding ions to basic CDMch. Surprisingly, the effects of metal ion additions were limited. Marginal improvements to *S. uberis* growth were seen following addition of Fe^{2+} and Mg^{2+}, whilst the effect of Mn^{2+} was more significant. These observations, whilst being notable for *S. uberis* strain 0140J, were barely evident however, when strain 20569 was similarly examined suggesting an even lower

Table 5.1: Changes in pH, absorbance and cell count during growth of strain 0140J in CDMcas.

Time (Hours)	pH	$OD_{600\ nm}$	c.f.u/ ml
0.00	7.49	0.026	4.8×10^{3}
4.20	7.53	0.029	5.2×10^{4}
24.00	7.39	0.047	5.7×10^{5}
25.40	7.41	0.045	3.4×10^{5}
28.30	7.43	0.045	3.5×10^{5}
30.30	7.50	0.042	3.4×10^{5}
46.00	7.01	0.138	8.2×10^{7}
47.30	6.83	0.205	1.7×10^{8}
48.50	6.64	0.242	3.2×10^{8}
50.15	6.44	0.317	2.9×10^{8}
52.00	6.03	0.409	3.8×10^{8}
53.30 *	5.37	1.222	3.7×10^{8}
54.30	5.06	1.987	1.5×10^{8}
71.00	4.30	2.335	1.5×10^{6}
73.30	4.35	2.311	2.0×10^{6}
76.30	4.24	2.262	2.5×10^{6}

* Point at which casein visually precipitated from the solution, corresponding with a vast increase in absorbance but no increase in c.f.u/ml.

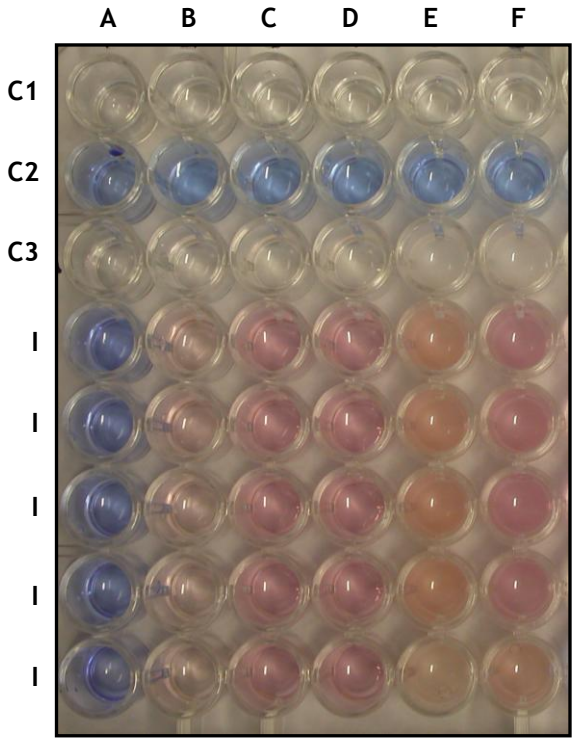


Figure 5.5: Utilisation of alamarBlue® to demonstrate viability of *S. uberis* 0140J cells in different CDM formulations. A stationary phase culture of *S. uberis* 0140J prepared in CDMch was used to inoculate test medium. Media only controls were loaded in row C1, media controls with alamarBlue in row C2, inoculated media controls with no alamarBlue in row C3 and inoculated replicates with alamarBlue in rows marked I. Media assessed were; CDM without metal ions (Column A); CDM with metal ions (column B); CDMch (column C); CDMch with metal ions (column D); CDMcas (column E) and CDMcas with metal ions (column F). Cells in all media are viable except CDM without metal ions only. The intensity of the colour produced is also an indicator of the cell viability.

requirement for metal ions (**Figure 5.6**). The basal RPMI medium included magnesium sulphate and potassium chloride, but no Fe^{2+} or Mn^{2+}, yet in the absence of hydrolysed casein, addition of Mg^{2+}, Mn^{2+} and Fe^{2+} alone or in combination, to the RPMI medium, failed to support the growth of *S. uberis* 0140J or 20569 even in the presence of a commercial amino acids solution (Sigma). It could thus be hypothesised that *S. uberis* has an essential requirement for specific casein derived peptides. Additionally, the hydrolysed casein used may be sufficiently contaminated with metal ions, such that no additional nutrients were required to permit growth when hydrolysed casein was present in the CDM.

Different chelating agents were added to CDMch in an attempt to restrict the concentration of metal ions that were present in the medium, so that more accurate observations of the effects of adding back individual ions could be made. Increasing the concentrations of the different chelating agents in the medium had minimal effect however on the growth of *S. uberis* strain 0140J. Growth rate and final absorbance values decreased only fractionally as the chelator concentration increased up to 1 mM (final concentration) and observations were remarkably consistent when using EDDA, 2'2'-dipyridyl or NTA (data not shown). Despite constituting part of the innate host immune response, addition of bovine lactoferrin to CDMch similarly had minimal effect on the growth characteristics of *S. uberis* 0140J (**Figure 5.7**). A concentration of 1 mg/ml had an observable effect on growth rate and final OD values, however, this was considerably higher than the concentration of 0.3 mg/ml bovine lactoferrin that was recently identified in mammary glands infected with *S. uberis* (Chaneton *et al.*, 2008) and this still did not seriously impact upon bacterial growth. The importance of individual metal ions could thus not be determined using this system.

Sufficient nutrients are available therefore in CDMch to support *S. uberis* growth without the need for additional metal ions, and growth is not inhibited by addition of metal ion chelators or natural antimicrobial chelating compounds. This suggests that either *S. uberis* has incredibly low requirements for metal ions, or that the bacteria efficiently utilises mechanisms to compete with chelators for ions that are available in the medium despite being complexed with a chelating agent.

5.2.3.2 Growth of *S. uberis* in defined medium pre-treated with Chelex-100®

Complete prevention of *S. uberis* growth could not be achieved using ion chelators and growth was permitted in defined medium with hydrolysed casein containing no supplementary ions or amino acids. As an alternative approach, a metal ion-restricted

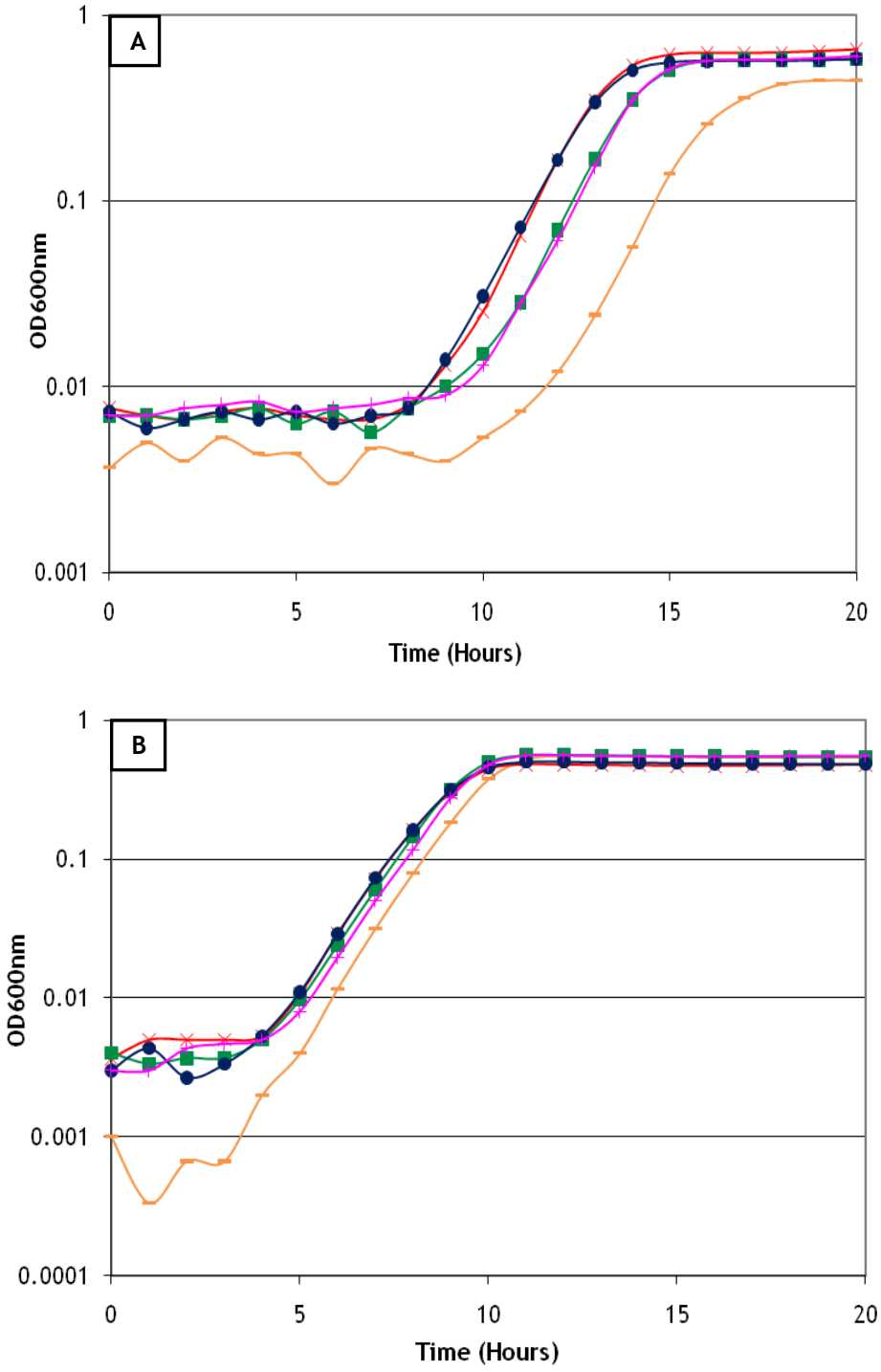


Figure 5.6: Effect of metal ions on growth characteristics of *S. uberis* 0140J (A) and *S. uberis* 20569 (B). A stationary phase culture prepared in CDMch was used to inoculate test media (CDMch) containing; no added metal ions (—▪—), 609 μM Mg (—+—), 45 μM Mn (—●—), 36 μM Fe (—■—) or Mg, Mn and Fe (—×—). Minimal effects are made on the growth of strain 0140J by addition of metal ions to the medium, and these effects are even less obvious for strain 20569.

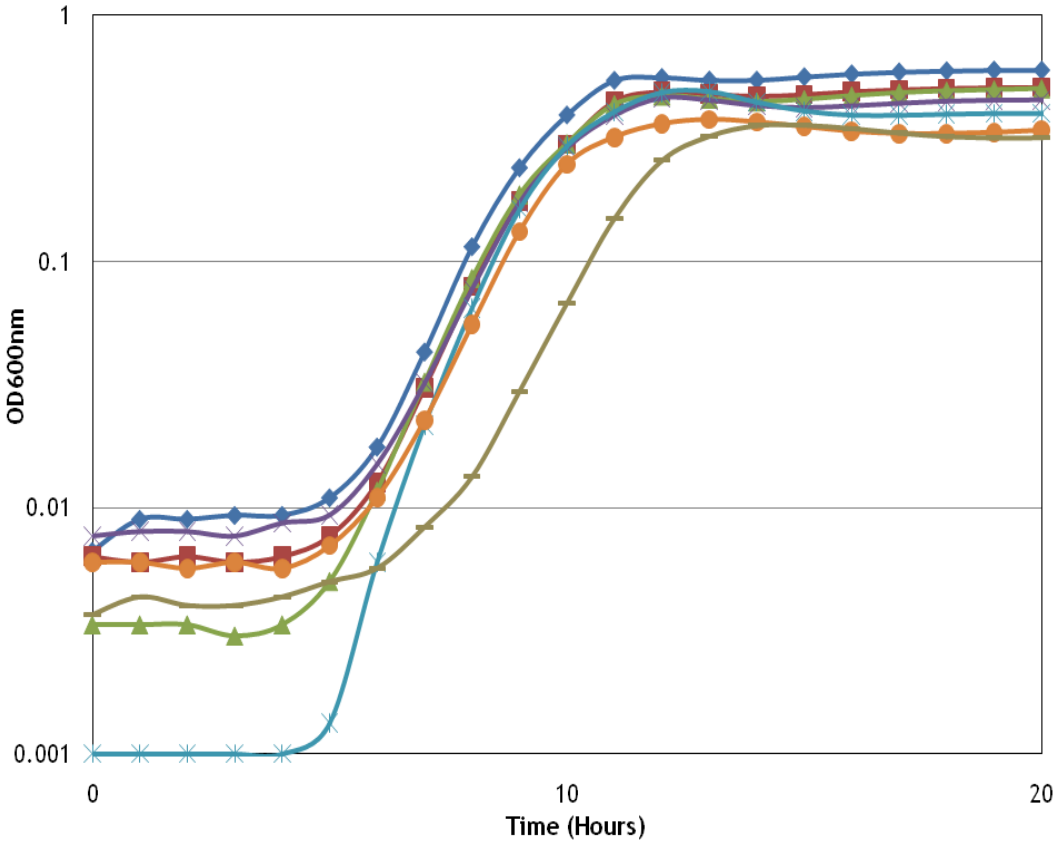


Figure 5.7: Effect of bovine lactoferrin on growth characteristics of *S. uberis* 0140J. A stationary phase culture prepared in CDMch with metal ions was used to inoculate test media (CDMch with Mn, Mg and Fe) containing; no lactoferrin ( ), 10 μg/ml lactoferrin ( ), 50 μg/ml lactoferrin ( ), 100 μg/ml lactoferrin ( ), 200 μg/ml lactoferrin ( ), 500 μg/ml lactoferrin ( ) and 1 mg/ml lactoferrin ( ). Growth rate and final OD values are somewhat reduced by addition of 1 mg/ml lactoferrin, whilst remaining concentrations have minimal effect on the growth of *S. uberis* 0140J.

medium was prepared using Chelex-100®, a styrene-divinylbenzene resin containing paired iminodiacetic ion groups which act as chelators for binding polyvalent metal ions. The Chelex resin, which has a particularly high sensitivity for divalent ions and transition metals was used to pre-treat CDMch. Ions bound to the resin were subsequently permanently removed by filtration of the matrix from the treated medium. A marked reduction in the final absorbance value was obtained following growth of *S. uberis* 0140J in filtered CDMch which had been treated with 2.5 % Chelex for 2 h. Incubation of *S. uberis* 0140J in CDMch pre-treated with 5.0 % Chelex for *ca*. 20 h did, however, reproducibly prevent bacterial growth completely (**Figure 5.8**). This result demonstrated that untreated CDMch does contain sufficient metal ions to support *S. uberis* growth, and that these elements, which are essential for growth, are successfully removed by Chelex treatment.

An approximation of the concentrations of ions required by *S. uberis* for proliferation in CDMch, and the relative importance of individual ions, was thus determined by individually adding ions back to medium pre-treated with 5.0 % Chelex. Increasing concentrations of single ions (Mg^{2+}, Mn^{2+} or Fe^{2+}) failed to stimulate growth of *S. uberis* 0140J. A combination of 300 μM Mg^{2+} and 50 μM Mn^{2+} was found however to support growth. Increasing the concentration of Mn^{2+} to 75 μM improved growth marginally, whilst 100 μM Mn^{2+} had no further effect on growth. Concentrations of Mn^{2+} greater than 100 μM retarded growth. Increasing the Mg^{2+} concentration to 400 μM also increased growth marginally. Combining Fe^{2+} with just Mg^{2+} or Mn^{2+} did not support *S. uberis* growth, but further optimisation of the ion concentrations did demonstrate that addition of 25 μM Fe^{2+} to Chelex-treated CDMch containing Mg^{2+} and Mn^{2+}, did improve growth rate noticeably (**Figure 5.9**). Iron appears therefore not to be essential for *S. uberis* proliferation despite improving growth rate.

Despite optimisation, growth of *S. uberis* 0140J in Chelex-treated medium remained noticeably lower than in untreated CDMch. As Chelex treatment removes polyvalent metal ions it seems likely that additional ions, such as zinc, are required in trace amounts also, to provide the optimum environment for growth. Supplementation of CDMch which had not been Chelex-treated with the optimal concentrations of ions (400 μM Mg^{2+}, 100 μM Mn^{2+} and25 μM Fe) had no effect on growth characteristics (**Figure 5.9**).

As concentrations of ions essential for *S. uberis* growth had been identified, the effect

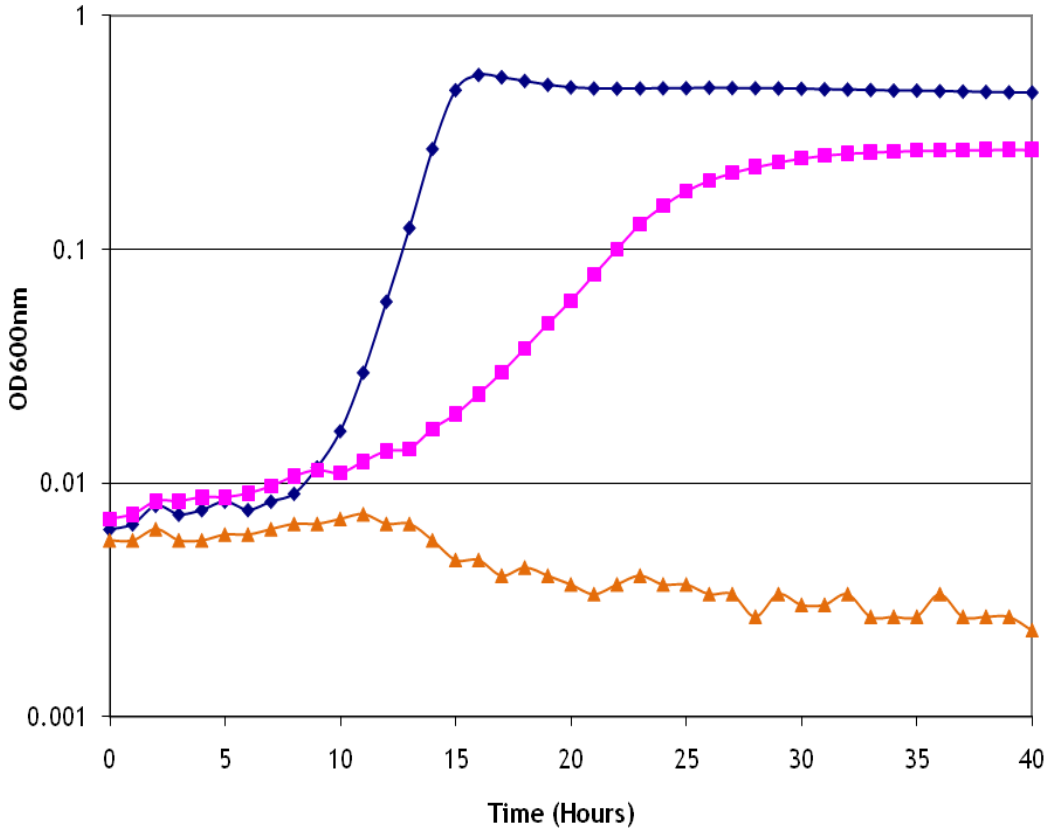


Figure 5.8: Effect of Chelex treatment on growth characteristics of *S. uberis* 0140J in CDMch. A stationary phase culture prepared in CDMch with no metal ions was used to inoculate test media; CDMch without Chelex treatment (—◆—), CDMch treated with 2.5 % Chelex and incubated for 2 h at RT (—■—) and CDMch treated with 5.0 % Chelex for *ca.* 20 h prior to filtration (—▲—). Treatment with 5.0 % Chelex completely prevents growth of *S. uberis* 0140J demonstrating the importance of metal ions for bacterial proliferation.

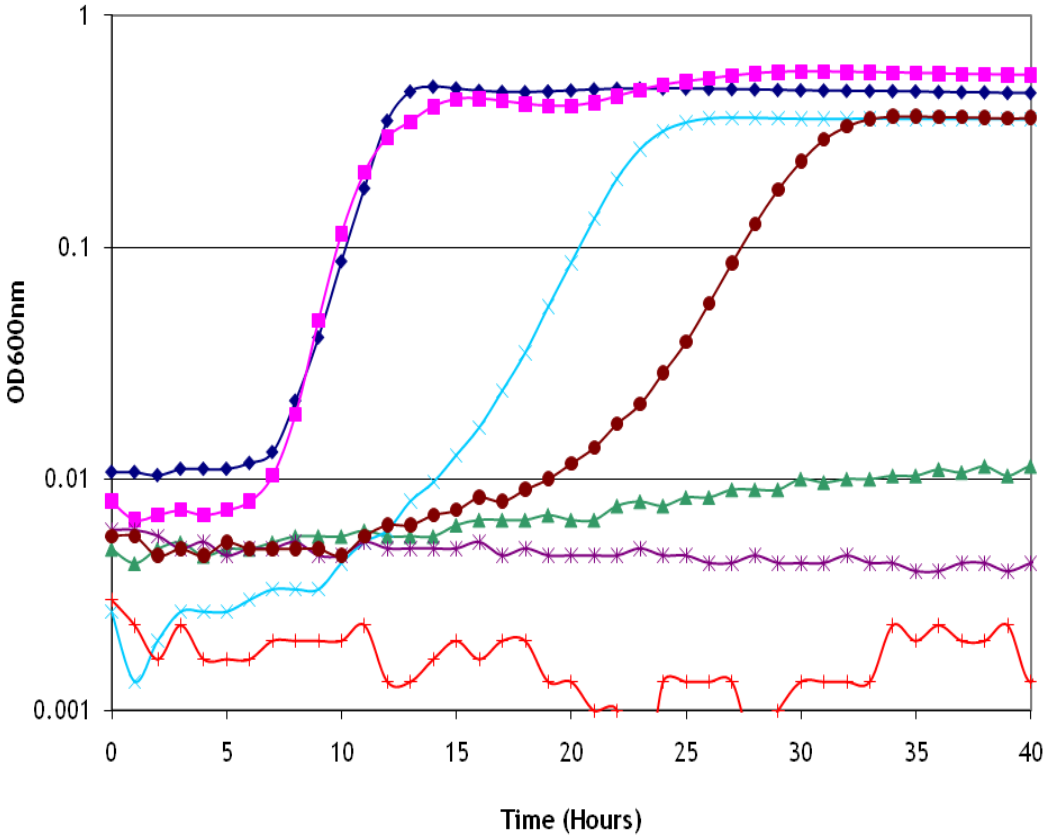


Figure 5.9: Growth characteristics of *S. uberis* 0140J following addition of ions to untreated and 5.0 % Chelex-treated CDMch. A stationary phase culture prepared in CDMch with no metal ions was used to inoculate test media; untreated CDMch ( ), untreated CDMch with 400 μM Mg, 100 μM Mn and 25 μM Fe ( ), Chelex-treated CDMch ( ), Chelex-treated CDMch with 400 μM Mg and 100 μM Mn ( ), Chelex-treated CDMch with 400 μM Mg and 25 μM Fe ( ), Chelex-treated CDMch with 100 μM Mn and 25 μM Fe ( ) and Chelex-treated CDMch with 400 μM Mg, 100 μM Mn and 25 μM Fe ( ). Complete restoration of *S. uberis* 0140J growth rate and final OD values to levels seen in medium prior to Chelex treatment was not achieved, but growth rate was clearly improved by addition of iron to the treated medium.

of chelating agents on growth in Chelex-treated medium supplemented with these essential ions only was examined. It was observed again, however, that chelating agents had only a limited effect on the growth of *S. uberis* 0140J. At a concentration of 1 mM, EDDA reduced the final cell density achieved by *S. uberis* 0140J noticeably, but lower concentrations had no marked effect (data not shown). This result supported the earlier hypothesis that *S. uberis* utilises unknown mechanisms to liberate essential ions from complexes formed by chelating agents.

5.2.4 Assessment of siderophore production by *S. uberis*

The chrome azurol S (CAS) assay (Schwyn and Neilands, 1987) demonstrates siderophore production by bacteria. A colour change in the medium corresponds to iron being liberated from the ferric complex of the CAS indicator dye by bacterial siderophores which have a higher affinity for the complexed iron. Siderophore production was initially examined in *S. uberis* reference strains 0140J and 20569. Following incubation at 37 ^{o}C for 48 h distinctive orange 'halos' were seen around the colonies of both strains, suggesting siderophore production. On further incubation halos became marginally clearer and were still evident after 7 days incubation. Equivalent observations were seen when bacteria were grown on agar plates, with or without 200 μM 2, 2'-dipyridyl prior to overlay with CAS medium (**Figure 5.10**). The CAS overlay assay was also used to demonstrate equivalent siderophore production by 100 % of 42 tested *S. uberis* mastitis isolates, which were predominantly from the Italian collection. The distinctive orange halos began to develop after 24 h incubation, developing further such that by 7 days the halos were very distinctive (data not shown). By incubating water aliquots of increasingly acidic pH (pH 9, 8, 7, 6, 5, 4 and 3) on top of CAS overlay media (poured into wells of a microtitre plate) for 1 week, it was shown that pH did not affect the colour of the medium and thus did not interfere with the assay (data not shown).

Siderophore production was further analysed using an adaptation of the CAS assay in a microtitre plate format. *S. uberis* reference strains were grown under low or high iron conditions (produced by presence or absence of 200 μM EDDA). Supernatant was then diluted into CAS medium and plates incubated for up to 24 h at RT. Although siderophore production was evident in strain 0140J, levels produced were very low and were in fact only observed in undiluted culture supernatant; strain 20569 produced no such colour change. Only a fractional difference in the intensity of siderophore production under high and low iron conditions was visible. To restrict iron further,

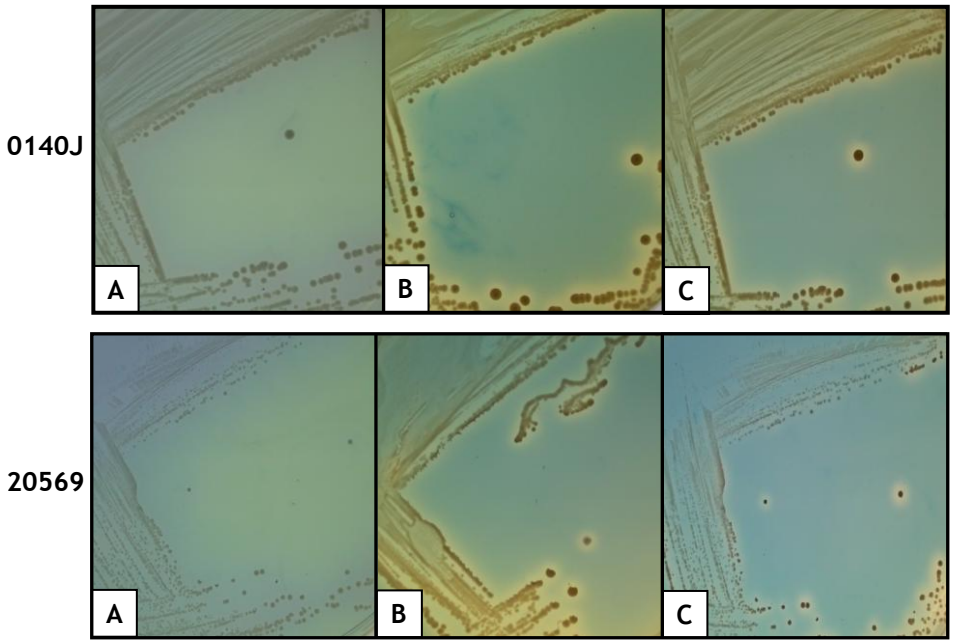


Figure 5.10: CAS overlay assay to demonstrate siderophore production by *S. uberis* reference strains. BHI agar with or without dipyridyl were streaked for single colonies using *S. uberis* strains 0140J and 20569. After 24 h incubation at 37 ^{o}C the plates were overlaid with CAS medium. Images represent observations made after; incubation of overlaid BHI agar plates for 1 day (A), incubation of overlaid BHI agar plates for 7 days (B) and incubation of overlaid BHI agar plates supplemented with 200 μM dipyridyl for 7 days (C). Orange halos around colonies indicate uptake of iron from the CAS indicator medium.

CDMch was treated with 2.5 % Chelex and the microtitre assay repeated using this medium and adding back either Mg^{2+} or Mg^{2+} and Fe^{2+} (at this stage optimisation of ion restricted conditions had not been completed). Siderophore production was, however, only evident in medium lacking additional Fe^{2+}. This change was again only observed in undiluted medium and some colour change was also visualised in control wells containing un-inoculated, undiluted medium suggesting that the medium may interfere with the assay.

5.2.5 *S. uberis* biofilm formation

The simple CRA assay was utilised to demonstrate slime forming ability in 100 % of UK, Italian and US *S. uberis* isolates tested (79 of 79), as well as reference strains 0140J and 20569. The production of distinctive black colonies by *S. uberis* strains was in clear contrast to the less obvious pink/red colonies formed by non-slime producing *Pasteurella multocida*, which was included as a negative control (**Figure 5.11**).

As all *S. uberis* isolates assessed were positive for slime production, a biofilm microtitre plate assay was utilised to explore biofilm production by these isolates in both defined and complex media. In this assay, biofilm formation was demonstrated by the adherence of cells to the surface of wells despite rinsing, these cells being subsequently stained with methyl violet to permit approximate quantification of aggregate biomass and thus biofilm formation. Whilst biofilm formation by *S. uberis* 0140J was absent following growth in BHI broth, biofilm formation was stimulated by growth in CDMch; alternatively, biofilm formation was seen following growth of *S. uberis* 20569 in both BHI broth and CDMch.

Biofilm formation was further visualised by placing glass cover-slips into wells of a 24 well plate containing the appropriate inoculated medium. After 48 h cover-slips were rinsed, stained and placed on a microscope slide permitting cells to be visualised. Comparisons were made between biofilms produced by strains 0140J and 20569 in BHI broth and CDMch under aerobic and anaerobic conditions (**Figure 5.12**). No biofilm formation by *S. uberis* 0140J was visible after growth under all conditions except aerobic growth in CDMch. Alternatively; biofilm formation was seen for *S. uberis* 20569 under all conditions, although this was enhanced considerably by growth in CDMch and growth in the absence of oxygen.

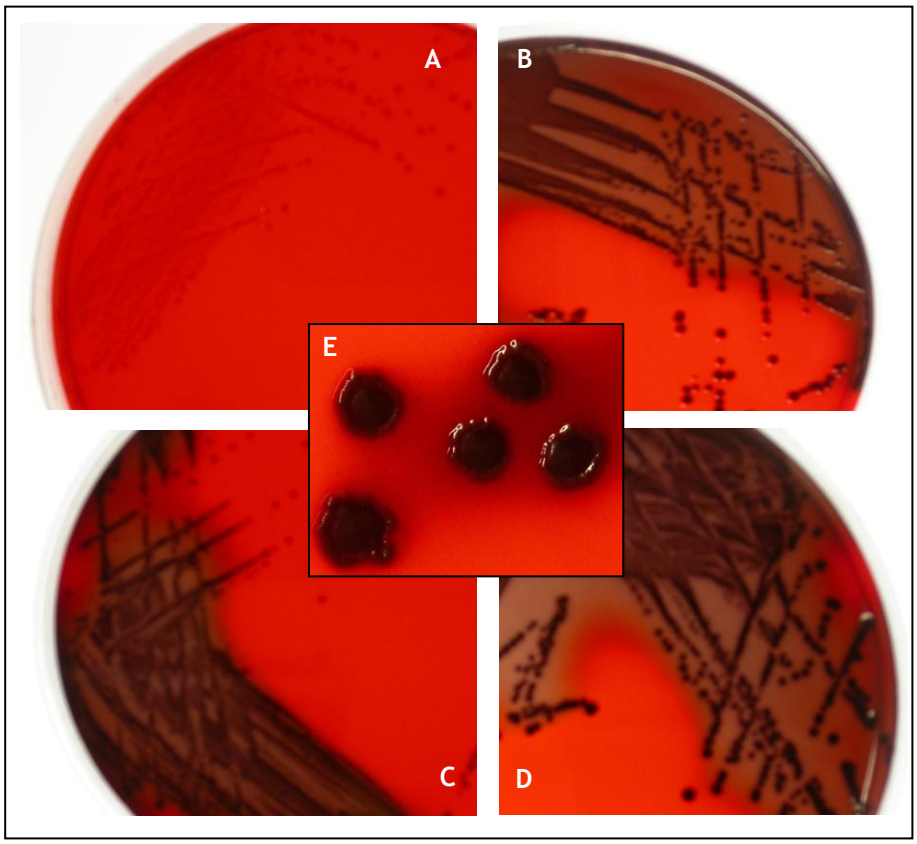


Figure 5.11: Detection of slime, a precursor to biofilm formation, using CRA plates. Indistinct pink colonies from negative control *Pasteurella multocida* (A); Black colonies of *S. uberis* strain 0140J (B); *S. uberis* UK mastitis isolate T1-60 (C) and *S. uberis* reference strain 20569 (D); an amplified image of the rough black colony type distinctive of a positive result (E).

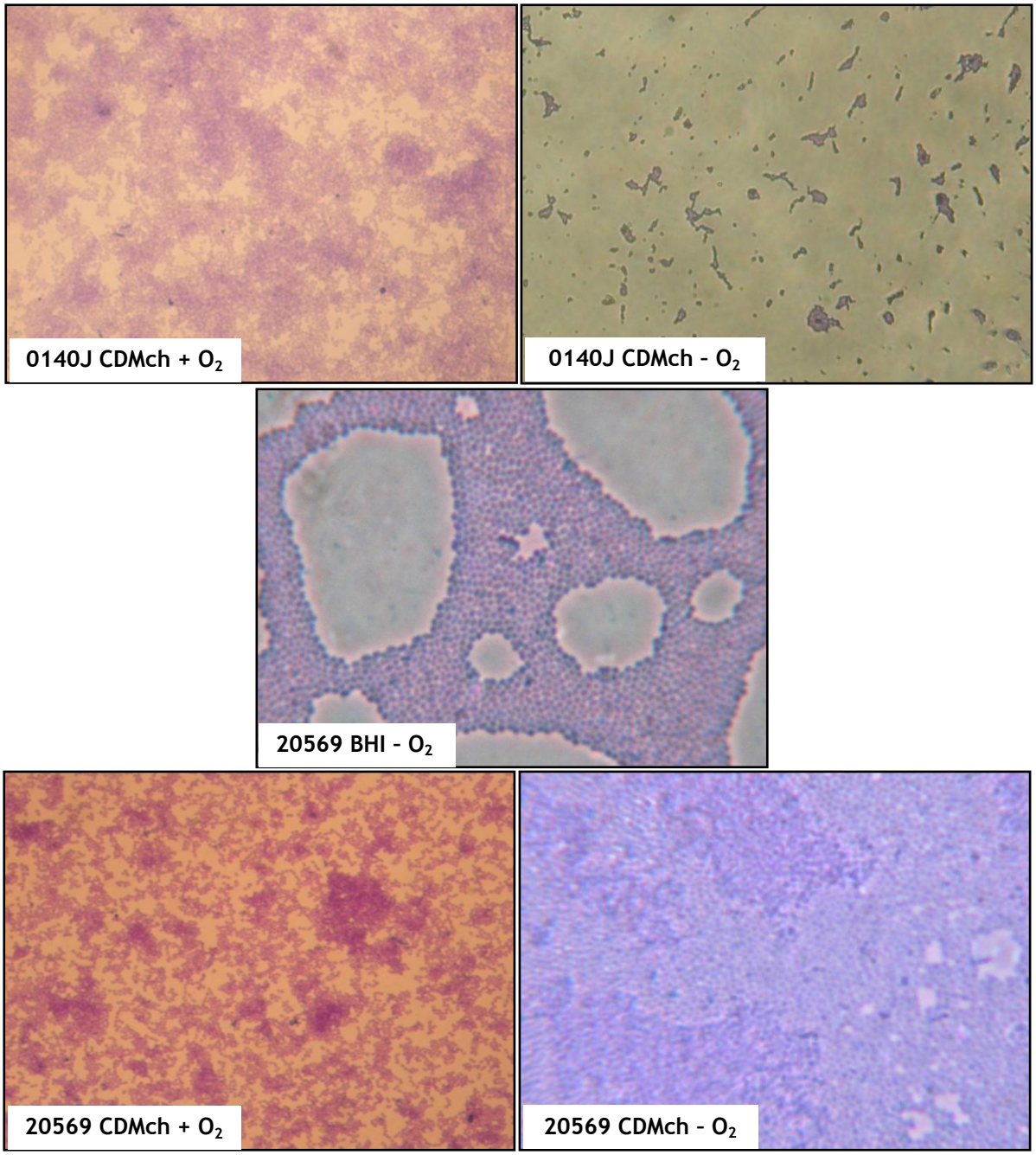


Figure 5.12: Microscopic visualisation of biofilm formation by *S. uberis* on glass cover slips. Biofilm formation by strains 0140J and 20569 following growth in CDMch under aerobic (+ O_2) or anaerobic (- O_2) conditions. Biofilm formation by strain 0140J following growth in BHI broth under both aerobic and anaerobic conditions were equivalent to the poor result seen for 0140J in CDM without oxygen. Biofilm formation by strain 20569 following aerobic growth in BHI was similar to that seen in CDM with oxygen. Biofilm formation by strain 20569 was enhanced by growth in defined medium and by exposure to anaerobic conditions during growth. Observations were made after static incubation of cultures for 48 h.

A diverse sub-panel of *S. uberis* strains were selected based upon epidemiological and MLST data (**Table 5.2**) and were further scrutinised for biofilm production using the microtitre plate assay. Initial experiments demonstrated that biofilm formation could be clearly observed after 24 h, with some increase by 48 h, but with minimal further change visible after 72 h (**Figure 5.13**). Additionally, biofilm formation clearly differed between strains, but was generally enhanced in most strains following growth in CDMch. Biofilm formation by all strains after 48 h of growth under additional conditions were subsequently determined and patterns of biofilm formation for each strain are summarised in **Table 5.3**.

Biofilm formation varied considerably depending upon the growth conditions employed. Strain I38 was the only sample which failed to produce biofilm under any of the conditions tested. Strains 20569, T1-60, T2-5, T2-67, I2, I6, I14, I26, I34 and 1:93 produced biofilms under all test conditions (**Figure 5.14**). In no case did any strain demonstrate the biofilm phenotype in BHI broth when this was not seen in CDMch. Addition of EDDA to BHI broth, or pre-treatment of BHI broth with Chelex did not affect biofilm formation (data not shown). Furthermore, in most cases there was no clear difference between degree of biofilm formation when iron was present or absent from depleted defined media, suggesting that iron has no obvious involvement in biofilm formation. It was observed however, that Chelex treatment of CDMch often had an effect on biofilm formation (essentially the same effect was observed both in the presence and absence of iron), stimulating biofilm formation by 4 strains and reducing biofilm formation by 12 strains. Notably, strains I10 and T2-36 only displayed the biofilm phenotype when grown in Chelex-treated medium.

Biofilm formation did not appear to be consistent amongst strains from the same farm, ST, CC or BURST group and ovine and bovine strains were equally capable or incapable of forming biofilms. Three strains (0140J and two non-persistent strains, T1-20 and T2-1) only displayed the biofilm phenotype following aerobic growth in CDMch; whilst seven strains (T1-22, T1-36, T2-10, T2-11, T2-53, T2-73 and I3) grew as biofilms in CDMch (with and without oxygen) but not in BHI broth. One strain (T1-43) was very notably affected by oxygen, as it failed to form biofilm in the presence of oxygen, yet biofilm formation was vastly stimulated in the absence of oxygen (when grown in either BHI broth or CDMch). Finally, two strains (I23 and I40) did not produce biofilms in BHI broth unless oxygen was removed, but did form biofilms in CDMch when oxygen was both present and absent, although biofilm formation was greater in CDMch when oxygen was limited. Anaerobic culturing of *S. uberis* in CDMch produced conditions most closely

Table 5.2: Sub panel of *S. uberis* strains further scrutinised using biofilm microtitre plate assay.

Strain	Country	Farm	Host [A]	Year	ST [B]	BCC [C]	DCC [D]	Info [E]
0140J	UK	-	B	1973	1	ND	5	Reference
20569	Germany	-	B	-	ND	ND	ND	Type strain
T1-20	UK	Crossman P	B	2000	330	12	-	NP
T1-22	UK	Crossman P	B	2000	331	1	143	P
T1-36	UK	Gale C	B	2000	336	14	-	P
T1-43	UK	Gale C	B	2000	337	1	-	NP
T1-60	UK	Hellier W	B	2000	345	5	-	NP
T2-1	UK	Hughes	B	2000	356	1	5	NP
T2-5	UK	Hughes	B	2000	5	1	5	P
T2-10	UK	Hughes	B	2000	6	1	5	P *
T2-11	UK	Hughes	B	2000	6	1	5	P *
T2-36	UK	Hughes	B	2000	5	1	5	NP
T2-53	UK	Hughes	B	2000	67	1	5	P
T2-67	UK	Paine E	B	2000	368	12	-	P
T2-73	UK	Paine E	B	2000	369	5	86	NTP
I2	Italy	Manca	O	2006	293	-	-	*pauB* +
I3	Italy	Zappaterreno	O	2006	294	-	143	*pauB* +
I6	Italy	Ferretti	O	2006	377	-	-	-
I10	Italy	Cricchi V	O	2006	299	5	-	-
I14	Italy	Tagliaferri	O	2007	Un	-	-	-
I23	Italy	Marini A.M.	B	2006	310	-	-	*pauB* +
I26	Italy	Greci	B	2006	304	1	-	-
I34	Italy	CRA	B	2006	Un	-	-	-
I38	Italy	Colognesi	B	2006	318	1	-	-
I40	Italy	Colognesi	B	2006	305	-	-	-
1.93	US	-	B	-	ND	-	-	-

[A] Host species was bovine (B) or ovine (O).

[B] Sequence types (STs) assigned by MLST or, strains not analysed (ND) or not assigned STs (Un).

[C] Group to which strain was assigned following BURST (BCC) analysis of UK and Italian strains, where a group was defined as a collection of isolates sharing at least 5 of 7 alleles.

[D] Database assigned CC (DCC) as defined upon submission to pubMLST database.

[E] Additional information; strains derived from a persistent (P), non-persistent (NP) or not truly persistent (NTP) infection (where NTP applies to samples derived from the same animal quarter over a period of time in which the animal was persistently infected, but in which MLST demonstrated that different STs were present over the infection period, suggesting cure of one infection and almost immediate re-infection with a different strain.

* Isolated from the same persistent infection but at different time points.

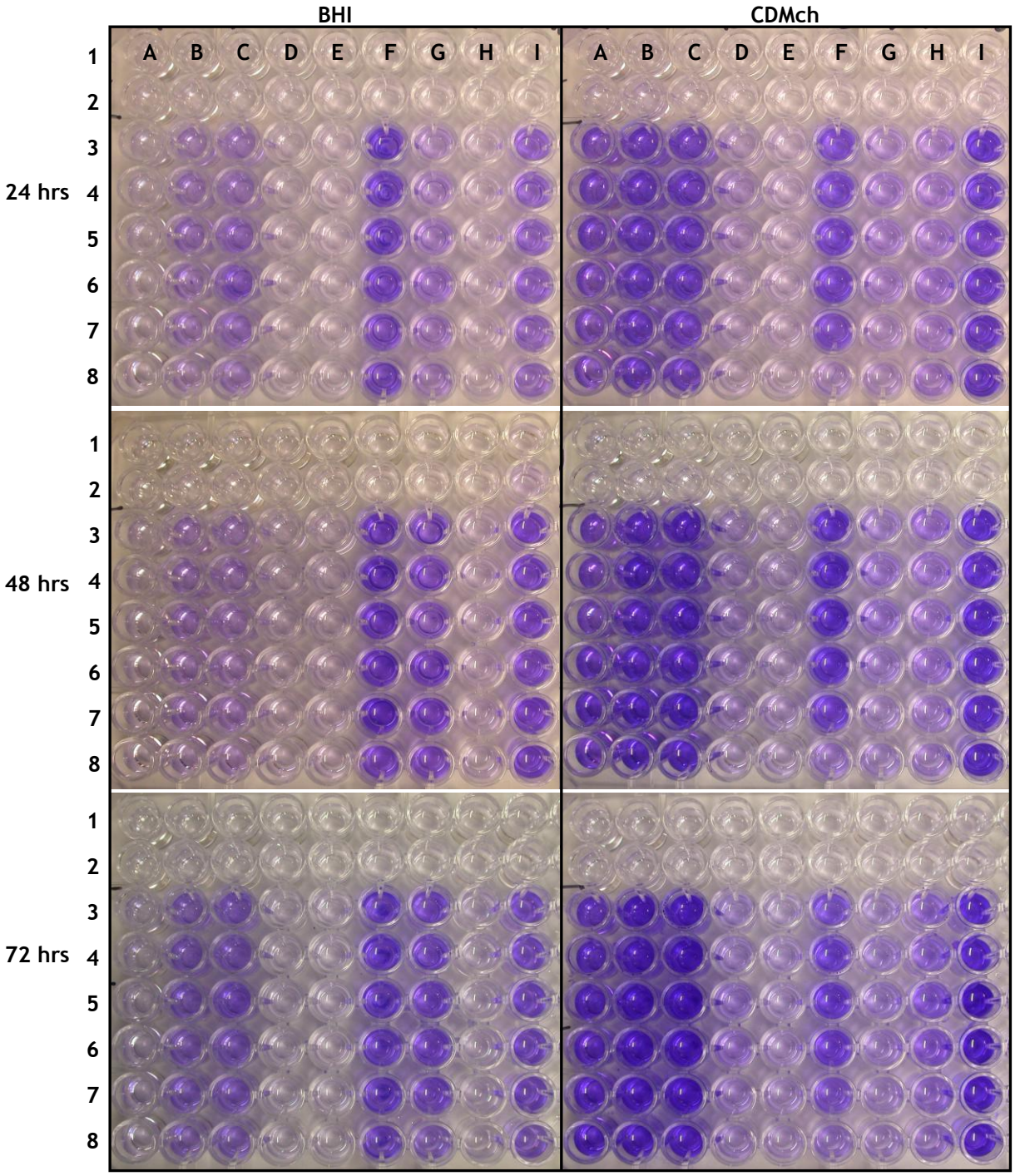


Figure 5.13: Visualisation of biofilm formation by 9 *S. uberis* strains following static growth in BHI broth or CDMch for 24 to 72 h using microtitre plate assay. Rows 1 and 2 contain media only controls whilst rows 3 to 8 contain replicates of medium inoculated with bacteria. Columns represent different strains: 0140J (A), 20569 (B), T1-60 (C), T2-10 (D), T2-11 (E), I2 (F), I3 (G), I40 (H) and 1:93 (I). Biofilm formation can be seen to increase marginally between 24 and 48 h, with marginal further increase in biofilm formation visible after 72 h. Biofilm formation was variable but for almost all strains, biofilm formation was higher after growth in CDMch over BHI broth.

Table 5.3: Biofilm formation by *S. uberis* strains following growth in different media.

Strain [C]	ST	Info.	Biofilm formation after 48 h [A, B]					
			BHI	BHI AN [D]	CDMch	CDMch Chelex + Mn, Mg [E]	CDMch Chelex + Fe, Mn, Mg [E]	CDMch AN [D]
0140J	1	Ref.	×	×	✓✓	✓	✓	×
20569	-	Ref.	✓✓	✓✓	✓✓✓	✓✓✓	✓✓✓	✓✓✓
T1-20	330	NP	×	×	✓✓	✓	✓	×
T1-22	331	P	×	×	✓	✓✓	✓✓	✓
T1-36	336	P	×	×	✓✓	✓	✓	✓✓
T1-43	337	NP	×	✓✓✓	×	×	×	✓✓✓
T1-60	345	NP	✓✓	✓✓✓	✓✓✓	✓✓	✓	✓✓✓
T2-1	356	NP	×	×	✓✓	✓✓	✓✓	×
T2-5	5	P	✓✓	✓✓	✓✓✓	✓✓	✓✓	✓✓✓
T2-10	6	P	×	×	✓	×	×	✓✓
T2-11	6	P	×	×	✓	✓✓	✓✓	✓
T2-36	5	NP	×	×	×	✓✓	✓✓	×
T2-53	67	P	×	×	✓✓	✓✓	✓✓	✓✓
T2-67	368	P	✓	✓	✓✓✓	✓✓	✓	✓✓✓
T2-73	369	NP	×	×	✓✓✓	✓✓✓	✓✓✓	✓✓
I2 *	293	*pauB* +	✓✓	✓✓✓	✓✓✓	✓✓✓	✓✓	✓✓
I3 *	294	*pauB* +	×	×	✓✓	✓	✓	✓
I6 *	377	-	✓✓	✓✓✓	✓✓✓	✓✓	✓✓	✓✓✓
I10 *	299	-	×	×	×	✓	✓	×
I14 *	-	-	✓✓	✓✓✓	✓✓✓	✓✓	✓	✓✓
I23	310	*pauB* +	×	✓✓	✓✓	✓✓	✓✓	✓✓✓
I26	304	-	✓✓	✓✓✓	✓✓✓	✓✓✓	✓✓✓	✓✓✓
I34	-	-	✓✓	✓✓✓	✓✓	✓	✓	✓✓
I38	318	-	×	×	×	×	×	×
I40	305	-	×	✓	✓	×	×	✓✓
1.93	-	-	✓✓	✓✓✓	✓✓✓	✓✓	✓✓	✓✓✓

[A] Biofilm formation was determined based upon absorbance values at 560 nm, calculated by normalising average values from 6 replica wells against average absorbance from two media only wells. Where experiments were repeated on another day, the value is the mean normalised absorbance from all experiments.

[B] Biofilm forming ability was graded as; No biofilm formation when absorbance was less than 0.1 (×), poor biofilm formation when absorbance was between 0.1 and 0.2 (✓), good biofilm formation when absorbance was between 0.21 and 1.0 (✓✓) and very good biofilm formation when absorbance was greater than 1.0 (✓✓✓).

[C] Strains were isolated from cows with the exception of those from sheep which are marked (*).

[D] Experiments conducted under anaerobic conditions (AN).

[E] Chelex treatment was used to deplete medium of all metal ions, prior to addition of specific ions to medium.

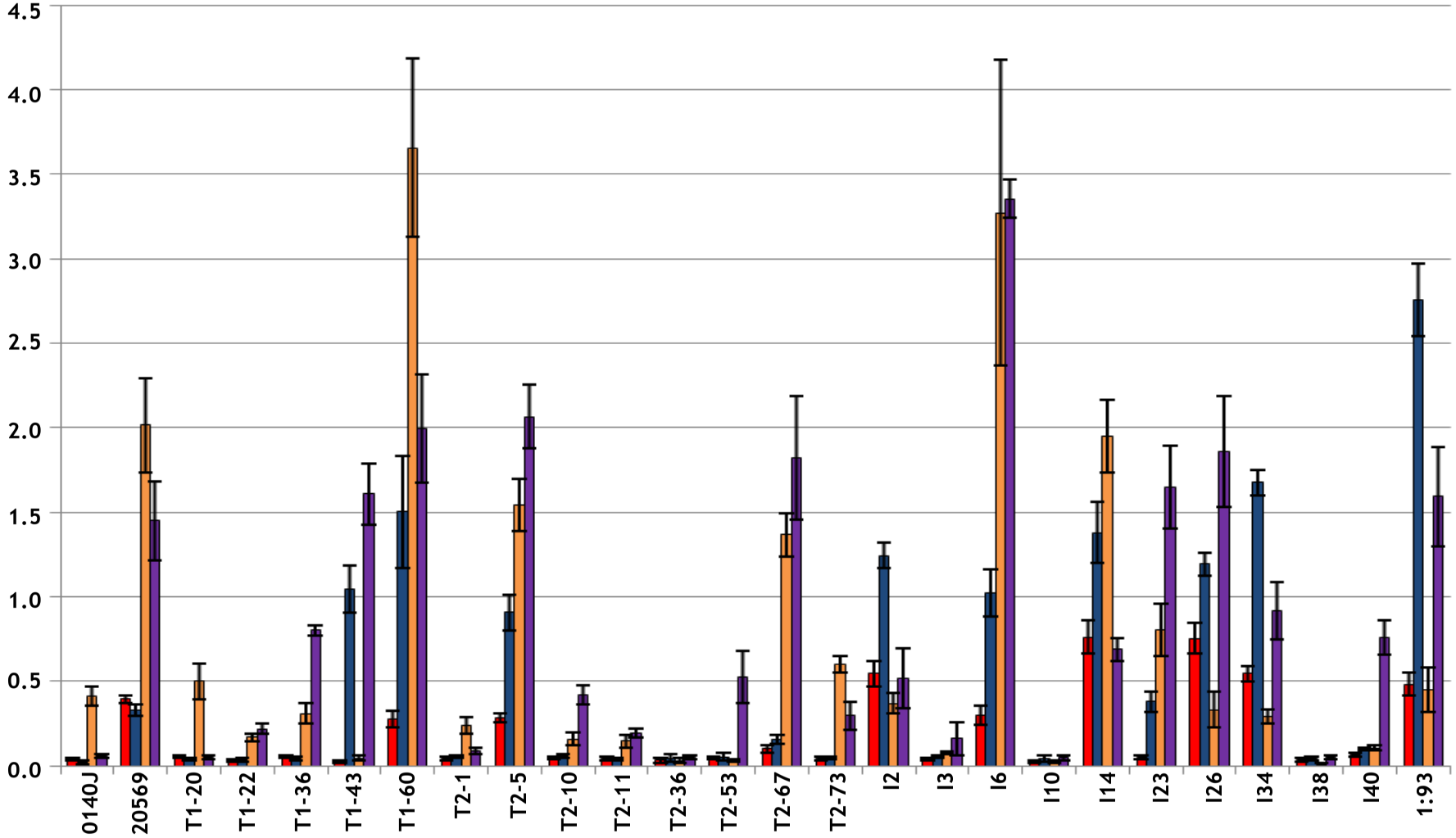


Figure 5.14: Biofilm formation by *S. uberis* strains. Absorbance at 560 nm (y axis) was the average value from six test wells normalised against the mean absorbance from two control wells containing the corresponding media only. Error bars are shown representing the standard deviation from mean results. Biofilm formation is shown for each strain following culture in; BHI under aerobic conditions (red columns), BHI under anaerobic conditions (blue columns), CDMch under aerobic conditions (orange columns) and CDMch under anaerobic conditions (purple columns).

reflective of the atmospherically and nutritionally limited status of the udder/milk environment. Under these conditions it was observed that all tested strains from persistent infections (7 of 7) produced biofilms (normalised OD value greater that 0.1), often in high quantities, whilst half of the strains from non-persistent infections (3 of 6) did not (normalised OD value less than 0.1); the *P*-value for this association (determined using Fisher's exact test) was 0.07. Whilst this value does not generally denote statistical significance, it does suggest a trend for persistent strains to form biofilms more readily under these conditions; given a larger test set, this observation may have been more apparent.

The relative ability for isolates T2-5 and T2-36 to form biofilms was of particular interest, since both isolates had been identified as sharing the same multi-locus ST, yet one was isolated from a persistent, and the other from a non-persistent infection, on the same farm. Under all conditions tested T2-5 produced much more biofilm than T2-36. Notably T2-5 displayed the greatest biofilm-forming ability of all tested strains after anaerobic growth in CDMch, with a corrected absorbance value of 2.06, whilst T2-36 showed the lowest (0.05). Clearly these strains do not share the same genotype (despite sharing identical alleles at 7 housekeeping loci) and the genetic heterogeneity of these strains was later confirmed by RAPD typing (presented in **Chapter 3**). Conversely, isolates T2-10 and T2-11 derived from the same persistent infection at different time points were shown by MLST and RAPD typing to be genetically identical. These strains also shared practically identical biofilm forming profiles. Both strains failed to produce biofilm in complex medium but did display the biofilm phenotype in CDMch.

5.3 Discussion

The use of a defined growth medium, in which all components and their corresponding concentrations are known, is an extremely useful tool for the characterisation of bacterial growth, and may identify factors which are important for survival *in vivo*. Such a medium may thus offer a direct means of providing information which may be pertinent to vaccine development. In this chapter, the production of a new *S. uberis* CDM that supported high levels of *S. uberis* growth was described and this novel medium was compared to a published recipe which had been used previously to culture *S. uberis* (Leigh and Field, 1991). Preparation of the published medium required, however, the individual addition of 46 different components, often in trace quantities, and this may have contributed to the inconsistent growth observations seen in this study when this medium was used (**Appendix 5**). In comparison, the novel CDM was easier and quicker to prepare due to the use of a commercial medium as the basal constituent, and subsequently, *S. uberis* growth appeared to be more consistent. Although not the specific objective of the work described in this chapter, the development of the CDM greatly facilitated subsequent work, and will be of great use to other researchers within our laboratory in the future. The defined medium developed in this study was intended to include the major factors associated with the *in vivo* milk environment, with respect to the carbohydrate (lactose) and metal ions provided, and the form of the amino acids (hydrolysed casein).

Growth rates of several different *S. uberis* isolates were characterised, and whilst some isolates (which all grew equally well in BHI broth) utilised the nutrients within the defined medium more efficiently than others, the CDMch nonetheless supported growth of all *S. uberis* isolates tested. Inter-strain growth variations in defined medium have similarly been observed with *S. aureus* and were attributed to differing absolute requirements for individual amino acids (Doherty *et al.*, 2006). The observations of growth variation in CDM between *S. uberis* isolates made in this study, may also imply an increased ability of some isolates to survive *in vivo*, as the defined medium more closely resembles bovine milk, and thus may be an indicator of 'fitness'. Genome sequencing of additional *S. uberis* strains (**Chapter 3**) suggested that despite a largely homologous genome, acquisition of additional genes; particularly those encoding transporters associated with nutrient acquisition, was not uncommon. This may explain the different abilities of strains to better utilise the nutrients present within the defined medium. For the work presented here, the defined medium provided a platform for the identification of elements important for *S. uberis* growth and observation of

related phenotypes. It has also been used concurrently within our laboratory as the culture medium for subsequent proteomic analyses of *S. uberis* strains (A. Jaglarz, unpublished data).

In the absence of hydrolysed casein, passage of *S. uberis* in CDM was not permitted, even in the presence of supplementary amino acids and metal ions. Previous research identified that eight amino acids were essential for *S. uberis* growth (Kitt and Leigh, 1997) and it was ensured that these were all present in the commercial RPMI medium utilised. The observation reported here was also in agreement with previous experiments which found that *S. uberis* growth was not permitted in medium supplemented with amino acids, either in their free form or present as peptides from milk, in the absence of plasmin-hydrolysed casein (Kitt and Leigh, 1997). The importance of specific casein derived peptides for the growth of *S. uberis* is thus clearly highlighted.

Bacteria rely upon the availability of peptides, often as a sole carbon or nitrogen source, and thus in turn upon proteins. To obtain these desired nutrients a system is required to degrade proteins and transport peptides into the cell, where peptidases further degrade these peptides to amino acids. Previously, *S. uberis* was found not to survive in medium supplemented with casein in the absence of additional peptides (Kitt and Leigh, 1997; Leigh, 1993). The activation of plasminogen (Leigh, 1993) and the acquisition of essential amino acids from plasmin derived casein peptides (Kitt and Leigh, 1997) have instead been demonstrated by *S. uberis*. This mechanism has thus been hypothesised as a method by which essential nutrients from the udder environment are acquired. An *S. uberis* plasminogen activator, PauA has indeed subsequently been characterised (Rosey *et al.*, 1999), but mutation of the *pauA* gene did not prevent *S. uberis* from infecting the bovine mammary gland (Ward *et al.*, 2003), and by analogy did not prevent the mutant strain from acquiring the nutrients it required for survival. Furthermore, *pauA* deficient strains have been recovered from cows with mastitis (Khan *et al.*, 2003; Zadoks *et al.*, 2005a).

Utilising the defined medium developed in this study, it appeared that *S. uberis* was in fact capable of directly utilising casein to support growth. Growth rate was significantly slower than in medium containing hydrolysed casein, but after an extended lag phase of between 20 and 30 h, rapid growth was observed, and maximum absorbance values exceeded those obtained in BHI broth. Despite the *S. uberis* CDM (based on RPMI) being buffered with $NaHCO_3$, it was evident that the buffering capacity was not sufficient to

prevent a drop in pH from 7 to 4 (as would be expected in un-buffered media) during culturing. Significantly, it was subsequently shown that casein within the medium precipitated at pH values below 5, which had a knock-on effect of distorting absorbance-based determination of cell density. Determining *S. uberis* proliferation by colony counting, confirmed, however, that the organism, following an extended lag period (as is observed using absorbance-based growth measurement), did begin to grow; as growth progressed the pH of the culture changed, and once the pH dropped below 5, the casein precipitated. Significantly, for the pH to drop at all must have resulted from *S. uberis* metabolic activity (primarily from the production of lactic acid during lactose metabolism). According to colony counts, the bacterial growth reached stationary phase and then began to decline, shortly after the precipitation of casein, suggesting that the precipitated casein was no longer available to support *S. uberis* growth.

It is unclear whether the pH-related alteration of casein solubility is a deliberate approach employed by *S. uberis in vivo* or an accidental side-effect of normal metabolic processes. Certainly, it seems hard to reconcile the fact that loss of casein solubility would result in the protein becoming un-accessible to the organism; however, it is not clear at this stage whether casein is also degraded when the pH changes and furthermore whether *S. uberis* employs any additional mechanisms which permit the uptake of casein-derived peptides. The use of a cell-wall associated protease has been shown to be involved in the utilisation of casein by the related lactic acid-producing organism, *L. lactis*. Furthermore, a transport system, encoded by the *oppDFBCA* genes has been shown to take up specific peptides into the cell (Detmers *et al.*, 1998; Tynkkynen *et al.*, 1993). It is unclear at this time whether a similar proteolytic enzyme exists in *S. uberis*, but reliance upon this type of system might explain why the results of this study suggest that specific casein-derived peptides are required to support growth, despite the presence of excess amino acids. Certainly, genes homologous to *oppA* and *oppF* of *L. lactis* have already been identified in *S. uberis* and were shown to be up-regulated during growth in milk. Furthermore, mutation of these genes produced a marked, but not complete, reduction in growth (Smith *et al.*, 2002; Taylor *et al.*, 2003).

The *L. lactis* extracellular serine protease (PrtP) has been shown to be responsible for casein degradation (Juillard *et al.*, 1995; Visser *et al.*, 1988). Interestingly, no such *S. uberis* proteinase has been definitively identified; however, research in our laboratory has identified two extracellular proteinases, C5a peptidase and a serine protease, that were conserved amongst mastitis isolates (A. Jaglarz, unpublished data). Further

research into these proteases is clearly required, which may lead to the demonstration of their role in the breakdown of casein. One possibility that exists is that the degradation of casein by an *S. uberis* PrtP-related protease may initiate immediately upon entry of the organism into a milk-containing environment. The time required to degrade sufficient amounts of casein to support growth may in fact explain the excessive lag period observed during *S. uberis* growth in casein-containing CDM. Consequently, the pH-mediated precipitation of remaining casein may be an un-related factor; however, as discussed below, precipitated casein may provide an entirely different means of aiding *S. uberis* survival *in vivo*.

The existence of a direct mechanism of casein degradation could explain why a *pauA S. uberis* mutant was still capable of causing infection *in vivo* (Ward *et al.*, 2003). Nutrients, it seems, can be obtained by this organism via a plasmin independent route. Plasminogen activators remain highly conserved amongst diverse strains (**Chapter 3**), thus it seems likely that *S. uberis* utilises both plasminogen activators and alternative pathways to liberate peptides from the host environment for the facilitation of survival *in vivo*. As *S. uberis* is not reliant upon a single system, loss of one gene is therefore not completely detrimental to growth, even when nutrient availability is limited.

Other phenotypic observations drawn during the course of this project related to the metal ion requirements of *S. uberis*. Interestingly, supplementing the growth medium with lactoferrin was found to have no effect on the growth of the organism. Similarly, previous research has shown that *S. uberis* grows, albeit to a slightly reduced level, in the presence of apo-lactoferrin (Todhunter *et al.*, 1985) and resists the antimicrobial activity of bovine lactoferrin (Chaneton *et al.*, 2008). One of the main functions of lactoferrin is to bind free iron, rendering it inaccessible to the microbial pathogen. Consequently, some pathogens have evolved mechanisms of obtaining iron from lactoferrin, while others have evolved to rely more heavily on the use of other metal ions (such as manganese) in place of iron, and a small amount of time was spent during this project investigating which may be true of *S. uberis*.

Treatment of CDM with Chelex-100 resin allowed a more accurate assessment of the requirement for specific divalent metal ions for *S. uberis* growth. Unsurprisingly, extensive Chelex treatment resulted in a complete abrogation of microbial growth in the treated medium. What was interesting, however, was the fact that, upon replenishing the Chelex-treated medium with Mn^{2+} and Mg^{2+} there appeared to be no requirement for Fe^{2+} for *S. uberis* growth to be supported. While iron is widely known to

be important for bacterial survival through its involvement in diverse processes such as oxygen and electron transport, energy production, and DNA synthesis, it must equally be tightly regulated, since via the Fenton reaction free iron (Fe^{2+}) is oxidised to insoluble Fe^{3+} producing hydroxyl radicals which, if over-accumulated, are highly damaging to biological macromolecules (Halliwell and Gutteridge, 1984; Touati, 2000). Some species such as *Borrelia burgdorferi* (Posey and Gherardini, 2000), *Lactobacillus* spp. (Elli *et al.*, 2000) and *S. suis* (Niven *et al.*, 1999) have similarly been shown to grow normally in the absence of iron and utilise alternative ions to catalyse essential reactions. This perhaps offers an answer as to why lactoferrin only has minimal effects on *S. uberis* growth. In the work presented here, growth experiments demonstrated that *S. uberis* was reliant upon Mg^{2+} and Mn^{2+} ions for proliferation in defined medium. Manganese has been shown, in other lactic acid bacteria, to substitute for biological roles played by iron (Winterhoff *et al.*, 2004). In this respect, disruption of the *S. uberis mtuA* gene prevents manganese uptake, prohibiting growth in milk and preventing *in vivo* infection (Smith *et al.*, 2003). Alternatively, Mg^{2+} is essential to maintain the integrity of ribosomes and the function of many enzymes. Despite the fact that iron did not appear to be essential for *S. uberis* growth, addition of Fe^{2+} to Chelex-treated medium, along with Mg^{2+} and Mn^{2+}, did improve the growth rate but not the final growth level. In this respect, the observations were similar to those made previously for *S. mutans*, whereby manganese and magnesium were shown to be essential for growth, whilst iron and zinc merely stimulated growth further (Aranha *et al.*, 1982). It is recognised, however, that it is not possible within the limits of the current study (with no direct analysis) to discount the presence of trace amounts of contaminating Fe^{2+} within the Mn^{2+} and Mg^{2+} salts used to supplement the CDM, which may have inadvertently contributed to *S. uberis* growth (although the reagents used were of the highest quality).

The observation that both Mn^{2+} and Mg^{2+} are required to support *S. uberis* growth is interesting, and may be used to inform studies into virulence and therapeutic design, since the targeting of associated transporters or binding proteins might offer valid targets for study. In this respect, as mentioned previously, a metal binding protein (MtuA) which transports manganese has already been identified in *S. uberis* and whilst this was required for growth it could not be used for a vaccine as the protein itself was not exposed on the outside of the cell, so neutralising antibodies could not gain access and bind to it (Jones *et al.*, 2004; Smith *et al.*, 2003). This clearly demonstrates, however, the importance of *S. uberis* proteins which bind or transport nutrients into the cell, and similar targeting of other transporters might identify a more successful

therapeutic target. Significantly, the use of bacterial ABC transporters for vaccines or antimicrobial targets has been the subject of review (Garmory and Titball, 2004) and metal binding lipoproteins of *S. pneumoniae* have been successfully used to protect mice against both carriage of the bacterium and development of invasive disease (Briles *et al.*, 2000; Brown *et al.*, 2001).

A common means by which microbes have evolved to acquire iron from their extracellular environment is through the production of low molecular weight iron-binding molecules known as siderophores. Work conducted during this project initially suggested that *S. uberis* may in fact be producing a functional siderophore molecule, since siderophore assays using CAS agar appeared to demonstrate that the organism was obtaining iron from the external medium, resulting in a characteristic colour change. The time taken to achieve the colour change was in excess, however, of what would normally be the case for most siderophore-producing organisms, whereby iron sequestration begins to happen almost immediately. It is therefore unclear whether the observed phenotype has been interpreted correctly, although it was demonstrated that a decrease in pH (as occurs during *S. uberis* growth) does not affect the colour of the CAS medium. Certainly, at this stage in the project the available *S. uberis* 0140J genome sequence was analysed for the presence of a genetic locus encoding the necessary siderophore-biosynthetic machinery. While nothing sharing direct homology with well characterised siderophore biosynthetic genes was identified, up to 5 regions within the genome were identified that could possibly be associated with the manufacture of a molecule which may be a novel siderophore. The apparent absence of a requirement for iron for *S. uberis* growth makes it unclear, however, why the organism would require a siderophore, if indeed one does exist. Despite the identification of siderophore transport machinery in *S. agalactiae* (Clancy *et al.*, 2006), until recently, streptococci were generally accepted to be devoid of siderophores. The first streptococcal siderophore, 'equibactin', was recently described, however, in *S. equi* (Heather *et al.*, 2008). Consequently, there is now a precedent for streptococci to possess siderophore biosynthetic apparatus, and it may be that the same is true of *S. uberis*. Certainly, phage and other mobile genetic-element driven horizontal gene transfer between streptococci would be a possible means of acquisition of such a phenotypic trait.

To further assess and hopefully quantify siderophore production by *S. uberis*, microtitre plate CAS assays were conducted. Unfortunately, the results of these experiments were equivocal, since only a low level of siderophore activity could be detected, which could

have derived from interference by the medium itself. Certainly, BHI broth is known to interfere with this assay at higher concentrations, so it is not unlikely that CDM could have similar effects. Since time was limited at this stage of the project, it was decided not to pursue the siderophore question further. Consequently, although these results offer a tantalising possibility of the existence of a siderophore in *S. uberis*, clearly further work is required. As iron was found not to be essential for growth, the apparent very low levels of siderophore production are perhaps unsurprising. Since in growth experiments iron was shown to improve growth rates, concurrent production of an iron-binding siderophore, while not essential for survival, could, however, play a role in enhancing the growth of *S. uberis*, especially when nutrients become more limited; potentially explaining the somewhat delayed colour change visualised on CAS agar. Alternatively siderophore production may be utilised by *S. uberis* to acquire alternative ions such as manganese, and as such the assay was not designed to identify such a molecule.

Perhaps the most interesting and relevant phenotype observed during the research performed for this thesis was the observation that some *S. uberis* strains are able to form biofilms. Using the CRA assay, all strains tested were shown to produce slime/exopolysaccharide, which is regarded as a precursor to biofilm formation. Subsequently, biofilm formation was quantified using a microtitre plate assay, and it was demonstrated that biofilm formation varied between strains, and was also dependent upon the growth conditions used. Clearly, the CRA assay does not offer the best means of predicting biofilm capability. The inadequacies of CRA screening for predicting biofilm formation have similarly been observed recently for strains of *S. aureus* (Croes *et al.*, 2009).

The production of biofilms is not a new observation among streptococci in general. Biofilm formation by *S. pyogenes* for example, was shown to be optimal in peptide-rich, carbohydrate-poor medium at 23 ^{o}C (Cho and Caparon, 2005). Furthermore, weak *S. pneumoniae* biofilms have been shown to be produced when the organism is grown in a complex medium, but much more significant, strong biofilm formation was stimulated in CDM (Moscoso *et al.*, 2006). The results presented in this chapter demonstrate that biofilm formation by most *S. uberis* strains was similarly greater following growth in CDM with hydrolysed casein peptides over complex BHI broth. Biofilm formation has thus been hypothesised as an additional strategy utilised by bacteria to survive in a nutritionally limited environment, as colonisation of a surface provides an increased opportunity to capture nutrients which accumulate at solid-liquid interfaces (Carpentier

and Cerf, 1993; Dunne Jr., 2002; Stanley and Lazazzera, 2004). Subsequent biofilm aggregation also permits central cells to survive in a dormant state where they require only minimal nutrients (Anwar *et al.*, 1992). Conditions that slow down bacterial growth, such as nutrient limitation, therefore favour biofilm formation by stimulating gene expression to switch the cell phenotype, such that survival in an inhospitable environment is promoted (Donlan and Costerton, 2002). During growth in complex medium nutrients are freely-available and cells thus require no specific adaptation, whilst in defined medium nutrients are limited so the cells adapt by forming biofilms as a survival strategy. Biofilm formation by *S. aureus* was, for example, inhibited by excess iron and stimulated in low-iron-containing medium (Johnson *et al.*, 2005). It has also been demonstrated though, that specific nutrients, such as magnesium ions, may also stimulate initial biofilm attachment (Song and Leff, 2006), highlighting the complexity of this process. Biofilm formation by *S. uberis* strains did not generally appear to be stimulated or inhibited by iron or Magnesium, as determined using Chelex-treated medium. While it was not possible within the time restraints of the work conducted for this thesis, it may be possible with further research to utilise optimised Chelex-treated CDMch to identify a specific chemical trigger that prompts *S. uberis* cells to alter their phenotype towards biofilm formation. It should be noted, that only preliminary stage biofilm formation was observed in this study; no attempt was made to distinguish between differentiated forms of *S. uberis* biofilms or to follow the biofilm life cycle.

In this study, biofilm formation in CDM by several strains was also influenced by the availability of oxygen. Similarly, biofilm formation by *S. gordonii* and *S. aureus* has been shown to be optimal under anaerobic conditions (Loo *et al.*, 2000; Ursic *et al.*, 2008). Anaerobic growth also regulated transcription of genes inducing the expression of a polysaccharide intercellular adhesin which has been reported to be required for cell to cell adhesion and biofilm formation in *S. epidermidis* (Cramton *et al.*, 2001). Again, biofilm formation appears to be stimulated directly in response to environmental conditions, and indeed low oxygen has been shown to up-regulate the expression of the *S. epidermidis* stress responsive factor stimulating biofilm formation (Cotter *et al.*, 2009). In this study, biofilm forming ability of *S. uberis* following growth in CDMch under anaerobic conditions was also most strongly correlated to *in vivo* persistence. All persistent strains were biofilm producers under these conditions, whilst just half of non-persistent strains formed biofilms. Furthermore, two strains defined as identical by MLST (ST 5) but which were derived from persistent or non-persistent infections from animals on the same farm, differed in their biofilm development, the strain from the persistent infection manifesting much greater biofilm-forming capacity than the non-

persistent strain. Despite apparent genetic similarities between these strains, the clear difference in persistence means that MLST has clearly not been sufficient to discriminate between these phenotypically heterogeneous isolates.

Biofilm formation therefore, at least circumstantially, does appear to contribute to *S. uberis* persistence although it does not guarantee it, as seen by some strains from non-persistent infections also eliciting a strong biofilm response; clearly this is an unsurprising observation, since successful colonisation and persistence of a pathogen within a host is a multifactorial process involving numerous equally-important factors. Certainly, the observations made with *S. uberis* strains are in keeping with the widely-held hypothesis that biofilm formation plays a role in the development of chronic *S. aureus* mastitis infections which resist the effects of both antibiotics and host factors (Aguilar *et al.*, 2001; Cucarella *et al.*, 2004; Melchior *et al.*, 2006a). Assessing the ability of persistent strains to produce biofilms *in vivo* would, more definitively, link these 2 observations.

It was intended that the sensitivity of *S. uberis* biofilms to antibiotics would be determined during this PhD project, to further demonstrate that biofilm formation plays a role in the development of chronic mastitis cases. Whilst it was clearly observed that planktonic *S. uberis* cells are sensitive to all tested erythromycin concentrations, unfortunately, resistance of biofilm cells to antibiotics (as measured by colony counts, biofilm cell density and cell viability) was inconsistent, and resistance displayed no correlation to the antibiotic concentration used (data not shown). Maintaining biofilms over a longer period, in which media changes are required, seemed in itself to produce inconsistent biofilm growth, resulting in the variable results observed upon subsequent addition of antibiotics to the growth medium. Time limitations prevented successful optimisation of this assay but initial observations of even just some cells surviving following antibiotic treatment suggests that biofilms do help protect *S. uberis* from antibiotics, supporting the hypothesis that biofilm formation plays a role in persistence of *S. uberis* in the mammary gland.

A dominant *S. epidermidis* ST was observed in human clinical isolates and the presence of genes encoding biofilm and resistance traits was greater amongst this ST than any other, such that these factors were hypothesised to have facilitated the establishment of this clone (Kozitskaya *et al.*, 2005). Similarly, an *S. aureus* CC was more highly-associated with strong biofilm formation than other clonal lineages (Croes *et al.*, 2009). At this stage, however, there is no evidence that *S. uberis* biofilm formation is

correlated to multi-locus ST or CC, and thus the high prevalence of ST 5 CC isolates may not be solely attributed to biofilm formation; in this respect, analysis of larger numbers of isolates would be required to draw a more definitive conclusion. Alternatively, as MLST defines homology based on just seven housekeeping genes and genome sequencing of *S. uberis* strains in this study (**Chapter 3**) identified plasticity of the genome, it is not unlikely that genes involved in biofilm formation are transferred between isolates, such that identity at housekeeping genes does not reflect carriage of this, or other, virulence-associated traits.

Data presented earlier in this thesis described the MLST and BioTyping analyses of *S. uberis* isolates. Although these typing techniques did provide some meaningful information with regards to the relationships between strains, it was not possible to address a major question which was whether or not there was a difference between bacteria isolated from either persistent or non-persistent infections. Consequently, the main objective of the work described in this chapter was to conduct an unbiased analysis of *S. uberis* isolates in order to identify phenotypes that may contribute to virulence, which may ultimately be found to be more frequently associated with persistent or non-persistent strains. Clearly, this objective has been successful, in that new insights into previously unreported phenotypes have been gained, in particular in relation to the ability of *S. uberis* to form biofilms. It is unfortunate that, due to issues of time and resource, no further progress could be made with respect to the analysis of the (putative) siderophore or casein utilisation phenotypes; however, this work will be pursued in the future by other researchers within the laboratory. It was decided that the ability to form a biofilm was the one identified phenotype that could be responsible for particular *S. uberis* strains persisting despite antibiotic therapy, and hence the research described in the following chapter was conducted in order to investigate this attribute further, specifically by beginning to unravel the molecular basis behind *S. uberis* biofilm production.

Chapter 6: Investigation of the molecular basis of biofilm formation by *S. uberis*

6.1 Introduction

As discussed in **Chapter 5**, observation of *S. uberis* growth *in vitro* led to the identification of several (putative) interesting phenotypes and the discovery that *S. uberis* is able to form biofilms. Biofilm formation by strains from persistent mastitis infections seemed to be greater than for non-persistent strains and was generally enhanced by growth in nutritionally-limited medium. Furthermore, the observation that two strains from distinct animals, but which shared the same ST, differed in their abilities to form biofilms, was of potential interest, since the strain producing the greatest amount of biofilm was associated with a persistent infection, while the other strain was not. As previously discussed, biofilm formation is becoming an increasingly well-recognised method by which bacteria resist the action of antibiotics and host factors, and may be particularly associated with the development of chronic conditions. Consequently, further understanding *S. uberis* biofilm production and the mechanisms permitting its development would allow a better understanding of the pathogenesis of this bacterium, and may also progress the development of alternative therapeutic or preventative treatments. In this respect, vaccines incorporating exopolysaccharide derived from biofilm-forming bacteria have already been shown to reduce the frequency and severity of subsequent *S. aureus* mastitis infections (Amorena *et al.*, 1994; Perez *et al.*, 2009). While a similar approach could conceivably offer a means of reducing *S. uberis* mastitis, the fact remains, however, that at this time there is no understanding of the means by which *S. uberis* forms biofilms. The aims of the work described in this chapter were therefore to investigate the molecular basis of biofilm formation, with a view to determining the contribution of specific genes, and hence gene products, to this phenotype.

Several distinct mutagenesis approaches have been described for bacteria, and have allowed associations to be made between genes and specific phenotypes. On the one hand, random mutagenesis procedures, whereby a transposon or insertion element integrates randomly into the bacterial chromosome, can be used to generate a library of individual mutants which may then be screened to identify a phenotype of interest; in this way the responsible gene(s) may also be identified. In contrast, targeted mutagenesis of specific genes may be conducted in order to confirm the role of suspect genes; in this protocol, genes (or large fragments of genes) are deleted by allele exchange, and the phenotype of the resulting isogenic mutant compared to that of the parent strain. Both random and targeted mutagenesis approaches address the same question, but from a different angle, and hence may be used in conjunction within the

same study. The plasmids pG$^+$host9 and pGh9:IS*S1* (the latter containing a bacterial insertion sequence promoting random-insertion into the chromosome), have been widely utilised to determine the functions of streptococcal genes. The pG$^+$host9 plasmid was, for example, utilised for allele replacement mutagenesis of *S. pyogenes*, permitting the production of a mutant which failed to produce streptococcal acid glycoprotein and was thus less able to invade and survive in epithelial cells (Degnan *et al.*, 2000). Allele exchange mutagenesis, using a deletion construct which was inserted into a pG$^+$host9 plasmid derivative, has also been used to determine the effects of deleting the *S. uberis lbp* gene (Moshynskyy *et al.*, 2003). Alternatively, random insertion of pGh9:IS*S1* into the chromosome of *S. suis*, identified the gene encoding a secreted nuclease (*ssnA*) that was highly associated with virulence (Fontaine *et al.*, 2004). A gene cluster responsible for the haemolytic ability of *S. agalactiae* was similarly determined through screening of a transposon generated mutant library (Spellerberg *et al.*, 1999). The specific function of many genes which have been speculated to play a role in the pathogenicity of *S. uberis* have yet to be assigned, however, the generation of mutants by transformation has recently been successfully demonstrated in this species. An *S. uberis* 0140J mutant library was generated in 2001, also utilising the pGh9:IS*S1* mutagenesis system, and this has permitted the identification of; three genes involved in hyaluronic acid capsule formation (Ward *et al.*, 2001), a mutant unable to activate plasminogen (Ward *et al.*, 2003), a mutant unable to survive in milk (Smith *et al.*, 2003) and a mutant unable to acquire amino acids from casein peptides (Smith *et al.*, 2002).

Transposon mutagenesis has also been successfully utilised to identify the genes that regulate and encode biofilm associated proteins in *S. aureus* and *S. epidermidis* (Cucarella *et al.*, 2001; Mack *et al.*, 2000). In the first instance, a polysaccharide intercellular adhesin (PIA) was demonstrated to be involved in *S. epidermidis* biofilm formation and this adhesin facilitated initial attachment and accumulation of cell aggregates (Mack *et al.*, 1994; McKenney *et al.*, 1998). Virulence of PIA negative mutants was also lower than that of wild-type strains in rat or rabbit infection models (Rupp and Fey, 2001; Rupp *et al.*, 2001; Shiro *et al.*, 1994). Three genes forming an operon were identified as those responsible for synthesizing PIA, a positively charged homo-polymer of N-acetyl-β-1,6- glucosamine (PNAG), and were designated the *icaABC* (intercellular adhesion) genes (Heilmann *et al.*, 1996; Mack *et al.*, 1996; Vasudevan *et al.*, 2003). A fourth gene, *icaD*, was also later identified at this locus, between, and overlapping the *icaA* and *icaB* genes, which increased the catalytic N-acetyl-glucosaminyltransferase activity of *icaA* (Gerke *et al.*, 1998). A differentially

transcribed putative regulatory gene (*icaR*) was also identified preceding *icaA* in the opposite orientation (Cramton *et al.*, 1999). It was later proven that IcaR negatively regulates biofilm formation by binding to the promoter site of the *icaA* gene (Conlon *et al.*, 2002; Jefferson *et al.*, 2003). Antibiotics interfered with the binding of IcaR to *icaA*, stimulating biofilm formation, and increasing the ability of the bacteria to survive (Jeng *et al.*, 2008). Ethanol was also found to repress *icaR* transcription increasing biofilm formation, however, increasing concentrations of NaCl or glucose (which also stimulated *ica* expression) did not affect *icaR* expression; these observations suggesting that the *ica* operon is regulated by multiple pathways depending upon the particular environmental pressure and is not controlled simply by *icaR* (Conlon *et al.*, 2002; Jefferson *et al.*, 2003).

Biofilm formation was highly correlated with coagulase-negative staphylococcal isolates from neonatal sepsis but poorly related to those from healthy subjects (de Silva *et al.*, 2002). Similarly, 85 % of *S. epidermidis* isolates from polymer associated septicemic disease contained *ica* genes, whilst these genes were present in just 6 % of healthy skin isolates (Ziebuhr *et al.*, 1997). The *ica* locus was identified, however, in all tested *S. aureus* and *S. caprae* strains, and while most of these isolates produced slime, not all produced biofilms (Allignet *et al.*, 2001; Cramton *et al.*, 1999; Vasudevan *et al.*, 2003), suggesting that harbouring these genes does not guarantee biofilm formation. Disruption of the *ica* locus, however, still resulted in a loss of *in vitro* biofilm forming ability by *S. epidermidis* (Heilmann *et al.*, 1996) and *S. aureus* (Cramton *et al.*, 1999) strains. Furthermore, insertion of the *ica* locus into commensal *S. epidermidis* strains permitted subsequent biofilm formation and invasive disease development in rats (Li *et al.*, 2005).

The *bap* gene encoding a biofilm associated protein (Bap), was also later identified in *S. aureus; bap* was absent from all clinical human isolates tested and present in just 5 % of bovine mastitis isolates, but, carriage of *bap* correlated strongly with biofilm forming ability (Cucarella *et al.*, 2001). A subsequent report, failed, however, to identify *bap* in any of the *S. aureus* mastitis isolates they tested (Melchior *et al.*, 2009). Genes with high homology to *bap* were however identified in several other *Staphylococcus* and *Enterococcus* species, and harbouring these genes also strongly correlated with biofilm formation in infection derived strains (Toledo-Arana *et al.*, 2001; Tormo *et al.*, 2005). Strains which were *bap* positive were also observed to be capable of forming biofilms even in the absence of the *ica* operon and thus using a process independent of PIA (Cucarella *et al.*, 2004; Tormo *et al.*, 2005). Serum antibodies to Bap were also identified in cows naturally infected with *S. aureus*, demonstrating that the production

of biofilm proteins by bacteria occurs *in vivo*, and that the host's immune system recognises these antigens (Cucarella *et al.*, 2004). Biofilm associated factors may therefore have potential as vaccine candidates.

Subsequent research has demonstrated that antibodies against the polysaccharide PNAG were produced in high numbers following immunisation with bacterins from strong biofilm producing *S. aureus* strains, and to a lesser extent following stimulation with cell free PNAG, but antibody production was not stimulated following immunisation with weak biofilm producing bacteria (Perez *et al.*, 2009). Animals vaccinated with strong biofilm forming *S. aureus* bacterins also elicited a strong antibody response following heterologous challenge with a biofilm-producing strain, and bacterial numbers and mastitis symptoms were all reduced in these animals (Perez *et al.*, 2009). In another study the use of strong biofilm producing *S. aureus* bacterins to vaccinate animals similarly resulted in increased host antibody titres and reduced bacterial numbers following subsequent heterologous challenge, although, in this case, clinical mastitis was not prevented (Prenafeta *et al.*, 2010). Furthermore, it was also identified that a single PNAG serotype was shared by all strong biofilm producing *S. aureus* isolates, demonstrating that, vaccination using this target was likely to offer cross protection against multiple heterologous strains (Perez *et al.*, 2009).

Diffusible signal molecules are frequently used by bacteria to communicate, allowing regulation of gene expression in relation to cell density, in a process termed quorum sensing. Quorum sensing (QS) is considered to play an important role in bacterial proliferation and virulence gene expression; un-surprisingly then, this system is also believed to play a significant role in bacterial biofilm formation. As early as 1986, an *S. aureus* gene regulator was identified by transposon mutagenesis which induced expression of a number of virulence determinants, such as the toxic shock toxin and staphylokinase (Recsei *et al.*, 1986). The accessory gene regulator (*agr*) system has since been well characterised in staphylococci and shown to encode an auto-inducing peptide (AIP) based signalling system that regulates expression of secreted and cell-surface proteins associated with virulence (O' Gara, 2007). Deletion of *agr* from *S. epidermidis* allowed increased expression of an autolysin and increased biofilm formation (Vuong *et al.*, 2003). Strains with different *agr*–types were also shown to differ in their biofilm forming ability (Melchior *et al.*, 2009). Many recent studies have attempted to completely define the pathways by which staphylococcal biofilms are regulated, but these have all concluded that several genes are involved in this process, some of which interact with, and others which are distinct from, the *agr* system (Cotter

et al., 2009; Johnson *et al.*, 2008; Kim *et al.*, 2008; Ulrich *et al.*, 2007). Whilst several regulatory models have been proposed, the exact mechanism(s) by which biofilm formation in staphylococci is activated, or repressed, remains to be precisely determined. It is clear however, that biofilm formation is highly dependent upon environmental as well as QS signals, and that growth in this state is regulated by the expression of a complex network of gene pathways.

Bacterial intercellular communication can also be facilitated by what has now been found to be a large family of density-responsive transcriptional factors; identified because they shared homology to the *lux* genes initially discovered in luminescent *Vibrio* bacteria (Fuqua *et al.*, 1994). These bacteria were found only to illuminate when the cell density reached a critical mass, and production of luminescence was subsequently attributed to the removal of a repressor from media and the expression of an activator (Eberhard, 1972). The gene encoding LuxS, an S-ribosylhomocysteinase which synthesises the AI-2 auto-inducer molecule has since been found to be conserved in bacteria from diverse phylogenetic groups (Fuqua *et al.*, 1994; Gao *et al.*, 2009; Lee *et al.*, 2006; Lyon *et al.*, 2001; Sun *et al.*, 2004b; Surette *et al.*, 1999); however, the mechanism by which A1-2 is detected in these species has yet to be identified as it is distinct from that used by *Vibrio* bacteria (Sun *et al.*, 2004b). Recently, it has been shown that *luxS* regulates virulence gene expression in *S. aureus* and *S. epidermidis* independently of *agr* (Doherty *et al.*, 2006; Xu *et al.*, 2006). Regulation of *S. pyogenes* virulence factors by *luxS* has also been demonstrated (Lyon *et al.*, 2001). A *luxS* mutant of *S. mutans* displayed increased biofilm formation (Huang *et al.*, 2009) and inactivation of *S. epidermidis luxS* not only increased biofilm formation, but enhanced virulence in a rat model (Xu *et al.*, 2006). This was similar to the manner in which *agr* also regulates *S. epidermidis* biofilm formation in response to secreted signal peptides, suggesting a QS role for LuxS and highlighting the importance of intercellular communication for the regulation of biofilm formation (Xu *et al.*, 2006). Interfering with bacterial communication is thus being considered as a novel method by which bacterial infections can be combated, as reviewed recently (Njoroge and Sperandio, 2009). As therapeutics of this kind would not inhibit growth, it is less likely that the bacteria will develop resistance; instead this method would reduce bacterial pathogenicity (Njoroge and Sperandio, 2009).

In **Chapter 5**, biofilm formation was demonstrated by bovine and ovine *S. uberis* mastitis strains from the UK and Italy, and there was a trend, under certain conditions, for the biofilm phenotype to be associated with strains from persistent infections. It has

recently been demonstration that bacterins from biofilm producing bacteria have potential value as vaccine candidates (Perez *et al.*, 2009). To the author's knowledge there has been no published report describing biofilm production by *S. uberis* and thus currently no *S. uberis* gene products have yet been described as playing a role in biofilm formation. The main aims of this chapter were therefore to attempt to identify genes responsible for biofilm formation, using targeted or random mutagenesis protocols.

6.2 Results

6.2.1 Identification of potential *S. uberis* biofilm genes

The mastitis pathogens, *S. aureus*, *S. epidermidis* and *E. faecalis* have all been shown to produce biofilms, and growth as a structured community is believed to contribute to the development of chronic infections with these pathogens. In **Chapter 5** it was demonstrated that several different *S. uberis* mastitis strains were also capable of forming biofilms *in vitro*. Staphylococcal biofilm formation has been well characterised, and PIA (synthesised by the products of the *ica* locus) and Bap have well characterised roles; QS compounds, such as ribosylhomocysteinase, also appear to be involved in regulating biofilm production. The recently published *S. uberis* 0140J genome was utilised to identify ORFs encoding products which shared homology to IcaA, IcaB, IcaC, IcaD and LuxS biofilm proteins of *S. epidermidis*; no Bap homologue was, however, identified.

The degree of homology between the proteins of *S. epidermidis*, *S. aureus* and *S. uberis* are detailed in **Table 6.1**. Unsurprisingly, much lower homology was observed between the translated products of the *S. uberis* genes and the putative equivalents from staphylococci, than between those of *S. epidermidis* and *S. aureus*. The functions of the staphylococcal proteins during biofilm formation are listed in **Table 6.2** in comparison to the known or predicted roles of the *S. uberis* homologues. Interestingly, the *S. uberis* IcaA homologue was identified as hyaluronan synthase, required for *S. uberis* capsule synthesis. The IcaB and LuxS homologues alternatively, were similarly defined as polysaccharide deacetylase and S-ribosylhomocysteinase proteins respectively, sharing the same predicted functions as the *S. epidermidis* products. The remaining homologues were simply described as membrane proteins with no suggested functions assigned at this time. The *ica* genes of many staphylococcal species lie within an operon (*icaADBC*) and are co-transcribed from a single promoter (Allignet *et al.*, 2001; Cramton *et al.*, 1999; Heilmann *et al.*, 1996). In *S. uberis* however, the genes transcribing the homologous proteins are well distributed throughout the genome (**Table 6.2**) and are thus unlikely to be co-transcribed (although the involvement of a common regulator of gene expression may not be out of the question). Genes transcribing the *S. uberis* Ica homologues are forthwith termed *hasA* (IcaA homologue), SUB 0809 (*icaB* homologue), SUB 1487 (*icaC* homologue), SUB 0701 (*icaD* homologue) and *luxS* (*luxS* homologue).

Table 6.1: Comparison of the homology between proteins associated with biofilm formation in *S. epidermidis* to equivalent regions identified in *S. aureus* and *S. uberis*.

	Biofilm associated protein				
	IcaA	IcaB	IcaC	IcaD	LuxS
S. epidermidis					
Query sequence ID [A]	AAN17771	AAN17773	AAN17774	AAN17772	EFA87239
Protein length	412 aa	289 aa	355 aa	101 aa	156 aa
S. aureus					
ID of homologous sequence [A]	ZP06340663	CAQ51099	ZP06325781	YP042086	NP372658
Protein length	412 aa	290 aa	350 aa	101 aa	156 aa
Identity to *S. epidermidis* (aa)	79 %	65 %	75 %	59 %	91 %
Sequence coverage	100 %	92 %	98 %	99 %	100 %
S. uberis					
ID of homologous sequence [A]	YP002562971	YP002562144	YP002562776	YP002562040	YP002562697
Protein length	417 aa	307 aa	332 aa	825 aa	160 aa
Identity to *S. epidermidis* (aa)	25 %	29 %	21 %	36 % [B] 50 % [B]	38 %
Sequence coverage	53 %	72 %	91 %	30 %	92 %
Locus tag	SUB 1697	SUB 0809	SUB 1487	SUB 0701	SUB 1399
Gene ID	*hasA*	None	None	None	*luxS*

Regions encoding proteins homologous to characterized biofilm associated proteins were identified in the genome sequence of *S. uberis* 0140J. Information was derived using BLASTP v 2.2.23+ (Altschul *et al.*, 1997) to search the non-redundant protein sequences database for the query sequence and selecting either *S. aureus* or *S. uberis* as the target organism (with aa denoting the number of amino acids in the translated protein).

[A] Amino acid sequence NCBI accession number.

[B] Homology of the *S. uberis* product to the *S. epidermidis* protein was restricted to two distinct regions at the start and the end of the *S. uberis* protein, giving an overall sequence coverage of 30 %.

Table 6.2: Location and annotation of *S. uberis* 0140J biofilm associated gene homologues.

S. epidermidis	*S. uberis*		
Biofilm protein and function	**Homologous protein**	**Gene ID [A]**	**Location [B]**
N-acetyl-glucosaminyltransferase, IcaA			
Production of N-acetyl-glucosamine oligomers	HasA, hyaluronan synthase	7392978	1678089 - 1676839
PIA synthesis deacetylase, IcaB			
Deacetylation of the poly-N-acetylglucosamine molecule	Polysaccharide deacetylase	7391739	789739 – 788819
PIA biosynthesis protein, IcaC			
Synthesis of longer oligomers and translocation to the cell surface	Membrane protein	7391166	1480445 - 1479381
Intercellular adhesion protein, IcaD			
Permit optimal activity of IcaA	Membrane Protein	7391679	677635 – 680109
S-ribosylhomocysteinase, LuxS			
Negative regulator of biofilm formation	LuxS, S-ribosylhomocysteinase	7391456	1396531 - 1397148

Genes with homology to *icaB* and *luxS* have similar predicted functions in *S. uberis*, whilst *icaA* is homologous to *hasA*, the capsule gene, and finally, *icaC* and *icaD* homologues have no known or predicted function.

[A] NCBI Entrez Gene ID number for *S. uberis* genes encoding Ica homologues.

[B] Location of genes within genome of *S. uberis* 0140J (Acc. No. AM946015).

6.2.2 Conservation of putative biofilm-associated genes amongst *S. uberis* strains

The conservation of *ica* and *luxS* gene homologues amongst *S. uberis* mastitis strains was explored. Primers (*hasA*, SUB 0809, SUB 1487, SUB 0701 and *luxS* gene F & R) were designed using sequences identified from the *S. uberis* 0140J genome (AM946015) and PCR was used to amplify a large internal region of each gene. A single product of approx. 871 bp for the SUB 0809 gene was amplified from all tested isolates (n=216) suggesting that this gene may be of some importance (data not shown). Due to time and cost restraints, amplification of the remaining genes was instead explored using the same sub panel of 26 diverse strains from the total collection (**Table 6.3**) for which biofilm forming ability had been determined previously (**Table 5.3**). The *lux*S and SUB 1487 genes were also conserved amongst all 26 strains, whilst *hasA* and SUB 0701 were absent from 6 and 20 strains respectively. A *hasA* transcript also could not be amplified following RT-PCR analysis of mRNA extracted from these negative strains during the stationary phase of growth in BHI broth, demonstrating that this gene was also not expressed under these particular conditions (**Figure 6.1**).

Genetic diversity was further examined by sequencing the successfully amplified PCR products from each locus for all strains of the sub-panel. Forward and reverse sequences were aligned and then compared using MegAlign (Lasergene). Aligned sequences in text file format were copied into a non-redundant database programme which highlights identical sequences (NRDB program, Warren Gish, Washington University). This allowed alleles to be assigned to individual strains for each gene in a manner similar to MLST (**Table 6.4**). Allele sequences are listed in **Appendix 6**. Although not present in all strains, 85 % of the remaining *hasA* nucleotide sequences were identical. Alternatively, despite being conserved amongst all strains, the nucleotide sequences at the SUB 0809 locus were almost all unique. At SUB 1487 and *luxS* loci, approx. half of the strains shared the same sequence, whilst the remainder were mostly unique. Gene profiles were compared to biofilm formation of these strains as determined in **Chapter 5** and it was revealed that *hasA* and SUB 0701 genes were not essential for biofilm formation, as strains lacking one or both of these genes were often strong biofilm producers. Phylogenetic trees were constructed in MegAlign based upon nucleotide sequences and trees were very different for each gene demonstrating recombination between the genes (data not shown). Interestingly, the phylogenetic tree constructed using the SUB 0809 gene sequences, highlighted a cluster of strains that were all poor biofilm formers (**Figure 6.2**). Further observation of sequence

Table 6.3: Sub-panel of *S. uberis* strains in which the carriage of potential biofilm associated genes was studied.

Strain No.	Isolate ID	Strain Info	ST (CC)[A]	Biofilm[B]
1	0140J	Sequenced reference strain ATCC BAA-854	1 (5)	×
2	20569	Reference strain ATCC 9436	ND	✓✓✓
3	T1-20	UK, bovine, Farm A, animal A, non-persistent	330	×
4	T1-22	UK, bovine, Farm A, animal B, persistent	331 (143)	✓
5	T1-36	UK, bovine, Farm B, animal A, Persistent	336	✓✓
6	T1-43	UK, bovine, Farm B, animal B, non-persistent	337	✓✓✓
7	T1-60	UK, bovine, Farm C, animal A, non-persistent	345	✓✓✓
8	T2-1	UK, bovine, Farm D, animal A, non-persistent	356 (5)	×
9	T2-5	UK, bovine, Farm D, animal B, persistent	5 (5)	✓✓✓
10	T2-10	UK, bovine, Farm D, animal C, persistent	6 (5)	✓✓
11	T2-11	UK, bovine, Farm D, animal C, persistent	6 (5)	✓
12	T2-36	UK, bovine, Farm D, animal D, non-persistent	5 (5)	×
13	T2-53	UK, bovine, Farm D, animal E, persistent	67 (5)	✓✓
14	T2-67	UK, bovine, Farm E, animal A, persistent	368	✓✓✓
15	T2-73	UK, bovine, Farm E, animal B, non-persistent	369 (86)	✓✓
16	I2	Italy, ovine, Manca, Viterbo	293	✓✓
17	I3	Italy, ovine, Zappaterreno, Roma	294 (143)	✓
18	I6	Italy, ovine, Ferretti, Roma	377	✓✓✓
19	I10	Italy, ovine, Cricchi Valerio, Roma	299	×
20	I14	Italy, ovine, Tagliaferri, Roma	Unassigned	✓✓
21	I23	Italy, bovine, Marini A.M., Viterbo	310	✓✓✓
22	I26	Italy, bovine, Greci, Roma	304	✓✓✓
23	I34	Italy, bovine, CRA, Roma	Unassigned	✓✓
24	I38	Italy, bovine, Colognesi, Roma	318	×
25	I40	Italy, bovine, Colognesi, Roma	305	✓✓
26	1:93	US, bovine	ND	✓✓✓
27	-	Negative control – dH_20	-	-

This panel was designed to represent the diversity of the *S. uberis* mastitis collection in terms of origins and multi-locus STs.

[A] Sequence type assigned to strain using MLST and the CC to which the ST was assigned by the PubMLST database.

[B] Biofilm forming ability of the strain under anaerobic conditions in CDMch, as determined using microtitre plate assay, this being none (×), poor (✓), good (✓✓) or excellent (✓✓✓).

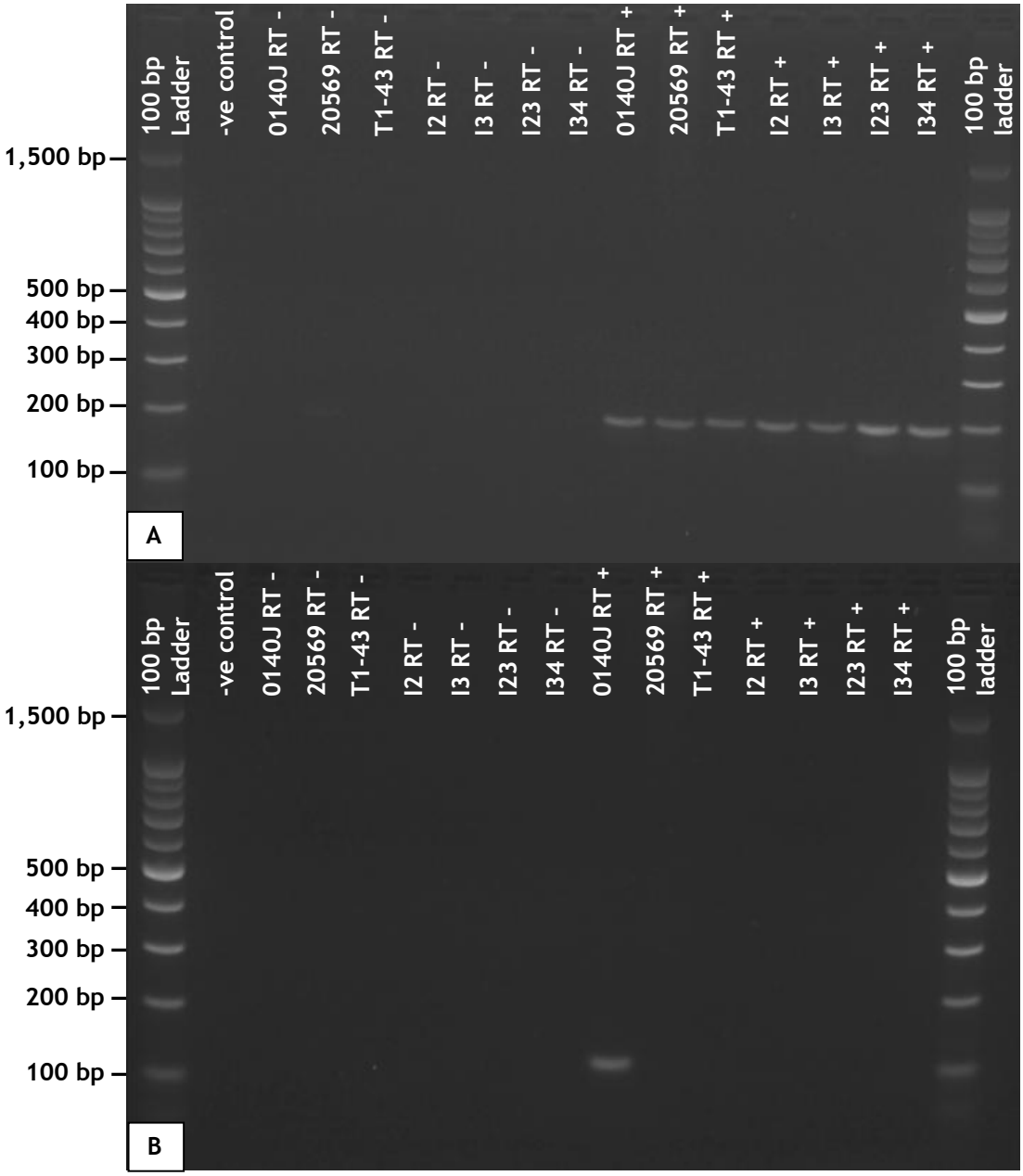


Figure 6.1: Reverse transcription PCR of *S. uberis* strains using *recA* F & R (A) primers or *hasA* 21 & 22 (B) primers to amplify *recA* or *hasA* transcripts respectively. Complementary DNA (cDNA) was prepared from strains in which *hasA* could not be amplified from gDNA. Extraction of RNA was conducted after 24 h of growth in BHI broth and RT conducted using the SuperScript kit (Invitrogen). RT negative controls were included to confirm that gDNA was successfully removed from the samples prior to amplification of cDNA. Strain 0140J in which *hasA* was amplified from gDNA was included as a positive control. All RT positive samples amplified a transcript for the housekeeping gene *recA*, whilst only 0140J amplified a *hasA* transcript.

Table 6.4: Allele and 'sequence types' assigned to *S. uberis* mastitis sub-panel based upon sequence data from *hasA*, SUB 0809, SUB 1487, SUB 0701 and *luxS* loci.

Strain	Gene					ST
	hasA	SUB 0809	SUB 1487	SUB 0701	*luxS*	
0140J	1	2	3	1	1	1
20569	Absent (4)	10	4	Absent (3)	2	2
T1-20	1	1	4	Absent (3)	9	3
T1-22	1	12	1	2	10	4
T1-36	1	11	1	2	1	5
T1-43	Absent (4)	4	1	Absent (3)	1	6
T1-60	1	5	1	Absent (3)	11	7
T2-1	3	2	1	1	12	8
T2-5	1	7	1	1	1	9
T2-10	1	2	1	Absent (3)	1	10
T2-11	1	2	1	Absent (3)	1	10
T2-36	1	3	1	1	1	11
T2-53	1	7	1	Absent (3)	1	12
T2-67	1	5	1	Absent (3)	5	13
T2-73	1	8	1	Absent (3)	1	14
I2	Absent (4)	15	5	Absent (3)	3	15
I3	Absent (4)	15	6	Absent (3)	4	16
I6	1	4	7	Absent (3)	5	17
I10	2	1	1	Absent (3)	5	18
I14	2	14	8	Absent (3)	6	19
I23	Absent (4)	9	9	Absent (3)	3	20
I26	1	7	1	Absent (3)	1	12
I34	Absent (4)	6	10	Absent (3)	7	21
I38	1	13	1	Absent (3)	1	22
I40	1	13	1	Absent (3)	8	23
1:93	1	10	2	Absent (3)	1	24

To allow determination of STs, the absent genes were arbitrarily assigned allele 4 at the *hasA* locus and allele 3 at the SUB 0701 locus. Only two pairs of strains shared identical profiles at all genes, one of these pairs (T2-10 and T2-11) was derived from a persistent infection of the same animal but on different dates whilst the other pair were unrelated and isolated from different countries.

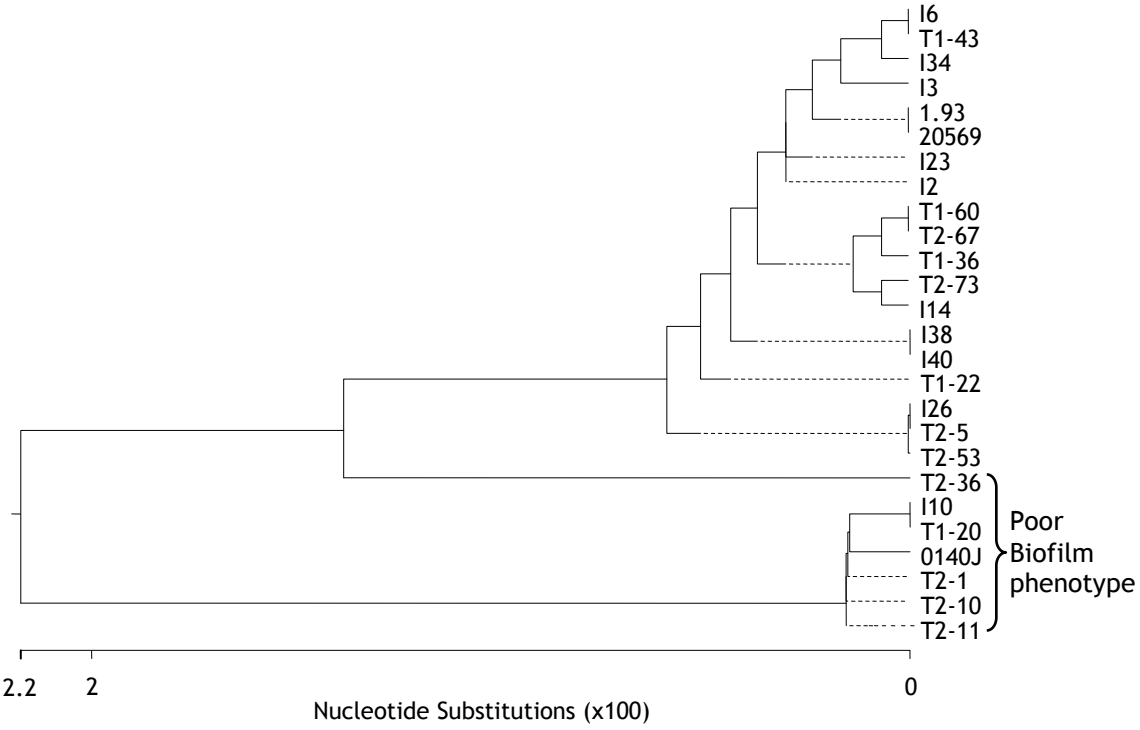


Figure 6.2: Phylogenetic tree demonstrating the heterogeneity in nucleotide sequences of *S. uberis* strains at the SUB 0809 locus. The phylogenetic tree was generated by aligning sequences from the SUB 0809 locus using the ClustalW method in the Lasergene MegAlign Software (DNASTAR, Inc.). A highly resolved cluster was visualised, and interestingly all the strains in this cluster produced poor or no biofilms when grown in defined medium under anaerobic conditions.

heterogeneity demonstrated that all poor biofilm formers, except I38, had distinctly different sequences at the SUB 0809 locus compared to the remaining strains which were better biofilm formers. The sequence of the SUB 0809 gene thus appears to correlate well to the biofilm phenotype. It was also observed that strains T2-5 and T2-36, both of which were assigned as ST 5 by MLST but which have very different biofilm profiles, differed at the SUB 0809 locus.

The *ica* gene homologues of *S. uberis* 0140J are spread throughout the chromosome, unlike the operon structure of *S. epidermidis* and *S. aureus*. Preliminary work was conducted to determine whether this gene organisation was conserved amongst additional *S. uberis* strains. Primers were designed to amplify the upstream and downstream flanking regions of the SUB 0809 gene. The upstream flanking region was amplified from both *S. uberis* 0140J and 20569 gDNA using primers SUB 0809 11 & 12 and sequencing confirmed identity. A PCR product of approx. 690 bp representing the predicted upstream flanking region was also amplified from all 216 of the *S. uberis* isolates tested (data not shown). Initial experiments failed, however, to amplify the downstream flanking region from strain 20569. Subsequent use of different primers (SUB 0809 13 & 14b) and optimised PCR conditions permitted amplification of a product which was around 200 bp smaller than the predicted fragment (and the product that was amplified from 0140J). Whilst time restraints prevented further investigations, it was clear however, that the region upstream of SUB 0809 was highly-conserved amongst all *S. uberis* isolates tested, but that some heterogeneity may exist downstream of this gene. Nonetheless, these observations further demonstrated that the *ica* gene homologues of *S. uberis* are unlikely to lie within an operon.

6.2.3 Optimisation of pG$^+$host9 transformation protocol

Transformation protocols for *S. uberis* (Moshynskyy *et al.*, 2003; Ward *et al.*, 2001) were utilised during this study and are described in detail in the methods section. Despite previous demonstrations of the transformability of *S. uberis*, in this study, strains 0140J and 20569 initially could not, however, be transformed with the pG$^+$host9 plasmid. This was consistent with the observation that transformation of some *S. uberis* strains appeared to be either inconsistent or not possible using published methods (Prof. J. Leigh and Dr. M. Fontaine, personal communication). In an attempt to increase the transformability of *S. uberis* cells, glycine was added to the culture medium used for obtaining competent cells, since over-incorporation of glycine into the Gram-positive cell wall has been shown to correspond to an increase in transformability in some bacteria, due to weakening of the cell wall (Ito and Nagane, 2001). The effects of

increasing glycine concentrations on the growth characteristics of *S. uberis* in complex medium were determined using the bioscreen C apparatus and permitted an estimation of the optimal glycine concentration for transformation. Growth rate and final OD values decreased steadily as the glycine concentration increased. The presence of 2.5 % glycine delayed growth by around 20 h and reduced final absorbance by about a third, whilst 3.0 % glycine was notably more detrimental to *S. uberis* growth (data not shown). Transformation of competent *S. uberis* 0140J cells prepared with 2.5 or 3.0 % glycine was attempted, but was unsuccessful. In addition, including an inhibitor of the Type I restriction modification system (TypeOne™ restriction inhibitor) in the transformation mixtures and assessing several different electroporator settings of voltage and current, did not permit successful transformation of 0140J.

In a further attempt to identify why transformation was unsuccessful, the pG$^+$host9 plasmid, which had been stored at -20°C for a considerable period of time, was digested with *Bsp*1286I to ensure it had not degraded. Using Clone Manager, the digestion products were predicted to be 2,368 bp and 1,384 bp respectively, and digestion did indeed produce DNA products of this size, implying that the plasmid was intact. Transformation of *S. uberis* 0140J remained unsuccessful; therefore a fresh stock of the plasmid was obtained from Prof. M. Kehoe (University of Newcastle), and was propagated in *E. coli* TG-1 Dev. A large-scale plasmid preparation was conducted, and the resulting DNA assessed by restriction endonuclease digestion with *Bsp*1286I. The expected digestion products were once again obtained, demonstrating that the new plasmid preparation was successful and further confirming the integrity of the original plasmid preparation (data not shown).

Newly prepared pG$^+$host9 (1 μg) was used to transform *S. uberis* 0140J competent cells. Surprisingly, in this experiment transformation was achieved with an efficiency of 8.6×10^{-5} %. In addition, and quite unexpectedly, during this experiment DNA from the original plasmid preparation was also used to successfully transform *S. uberis*, albeit at a slightly lower frequency (3.36×10^{-6} %). Use of competent cells which had been frozen and thawed prior to transformation was also shown not to be detrimental to the subsequent transformation (transformation efficiency 1.62×10^{-4} %) and the addition of glycine during competent cell preparation was not required for successful transformation.

Further optimisation identified that the use of between 1.0 and 1.5 μg of plasmid DNA was optimal for *S. uberis* transformation (% transformation 5.72×10^{-5} and 1.61×10^{-4}

respectively), and that addition of TypeOne restriction inhibitor did not improve transformation frequency further (% transformation 5.30×10^{-5} when using 1.0 μg plasmid DNA and 5.19×10^{-5} when using 1.5 μg plasmid DNA). The standard protocol subsequently used for transforming *S. uberis* cells with the pG^{+}host9 plasmid was thus the addition of 1.0 to 1.5 μg plasmid DNA to frozen competent cells prepared in BHI broth with yeast extract and hyaluronidase. Electroporation was conducted using electroporator settings of 2.4 kV, 100 Ω and 25 μF, and cells were then recovered in 10 ml BHI broth with yeast extract at the plasmid permissive replication temperature (28°C) for *ca.* 2 h.

6.2.4 Transformability of different *S. uberis* strains

To determine whether *S. uberis* 0140J was typical or atypical in terms of its ability to be transformed with plasmid DNA, competent cells from 12 additional, diverse *S. uberis* strains, were prepared and transformation attempted with pG^{+}host9 (**Table 6.5**). Transformation of eight of the twelve strains was achieved, and these strains displayed transformation efficiencies ranging from 5.13×10^{-5} to 9.23×10^{-3} %. Corroborating observations made earlier in this investigation, strain 20569 could not be transformed; also, erythromycin resistant colonies were not identified for strains T1-13, T2-10 and T3-23. Transformation efficiency clearly varied between experiments, as evidenced by the transformation frequency observed for strain 0140J (which was higher in a previous experiment). This could be attributed to a number of factors, including, the use of different batches of competent cells or different batches of media. In this experiment, the three Italian mastitis strains displayed the greatest ability to be transformed with the pG^{+}host9 plasmid, whilst 3 of 4 strains representing multi-locus STs 5 or 6 could not be transformed.

Plasmid preparations were conducted from a putative transformed, erythromycin resistant colony for each of the strains to confirm that the pG^{+}host9 plasmid had indeed been introduced into the bacterial cytoplasm. Plasmid samples from each strain were digested with *Bsp*1286I and analysed by electrophoresis. Despite the intensity of the resulting bands varying, the presence of two products of approx. the predicted size (2,368 bp and 1,384 bp) indicated that all these bacteria had been successfully transformed by the introduction of the plasmid into the cell (data not shown).

Table 6.5: Efficiencies with which *S. uberis* strains were transformed with pG$^+$host9.

Strain	Additional information [A]	c.f.u/ml			% Transformation
		Competent cells (Pre) [B]	Total (Post) [B]	Em^r (Post) [B]	
0140J	B, Genome sequenced / ST 1	4.60×10^{10}	1.58×10^{8}	8.00×10^{1}	5.13×10^{-5}
20569	B, Reference strain / ND	3.28×10^{10}	1.75×10^{8}	0	0
T1-13	B, UK, NP / ST 5	1.43×10^{9}	1.85×10^{8}	0	0
T1-36	B, UK, P / ST 336	5.57×10^{10}	1.83×10^{8}	4.50×10^{2}	2.46×10^{-4}
T1-43	B, UK, NP / ST 337	1.16×10^{10}	8.48×10^{7}	3.50×10^{2}	4.13×10^{-4}
T1-60	B, UK, NP / ST 345	1.73×10^{10}	1.87×10^{8}	1.90×10^{2}	1.02×10^{-4}
T2-10	B, UK, P / ST 6	1.58×10^{10}	1.23×10^{8}	0	0
T2-20	B, UK, NP / ST 6	2.70×10^{10}	1.61×10^{8}	1.55×10^{3}	9.63×10^{-4}
T3-23	B, UK, NP / ST 6	1.77×10^{9}	1.58×10^{7}	0	0
1:93	B, US / ND	3.55×10^{10}	1.41×10^{8}	2.00×10^{2}	1.75×10^{-4}
I2	O, Italy / ST 293	4.28×10^{10}	2.02×10^{8}	1.15×10^{4}	5.69×10^{-3}
I34	B, Italy / Unassigned ST	2.02×10^{10}	1.53×10^{8}	8.40×10^{3}	5.49×10^{-3}
I40	B, Italy / ST 305	2.47×10^{10}	1.55×10^{8}	1.43×10^{4}	9.23×10^{-3}

To determine the percentage transformation achieved, the number of colonies resistant to erythromycin (Em^r) was divided by the total number of viable cells in the sample (post electroporation), including antibiotic sensitive cells. Values are expressed as a percentage after multiplication by 100.

[A] Additional information regarding strain origins; strains derived from either bovine (B) or ovine (O) mastitis cases and originated from the UK, US or Italy. UK strains were also further characterised as deriving from a persistent (P) or a non-persistent (NP) infection.

6.2.5 Targeted mutagenesis of putative biofilm genes

Optimisation of a protocol for the transformation of *S. uberis* using pG$^+$host9 provided a platform for the production of *S. uberis* mutants using targeted allele replacement mutagenesis. To determine if the *ica* gene homologues identified in *S. uberis* were associated with biofilm formation, as they are in staphylococci, these genes were targeted for deletion from *S. uberis* 0140J.

The targeted mutagenesis protocol required primers to be designed for the amplification of approx. 1 kb regions upstream (*hasA* or SUB 0809 01 & 02) and downstream (*hasA* or SUB 0809 03 & 04) of the target gene. Small regions at the start and the end of the gene of interest were included in these fragments. To facilitate cloning, tags containing restriction endonuclease recognition sequences were added to the 5'-ends of the reverse primers for the upstream flanking regions and the forward primers for the downstream flanking regions. This allowed the resulting PCR products to be digested and ligated together easily, creating a product in which most of the target gene was absent, but which was flanked by regions homologous to the wild-type genome. The primers were also designed to prevent the resulting mutations inducing frame-shift, allowing subsequent changes in phenotype to be specifically attributed to the removal of the target gene only.

6.2.5.1 Construction of plasmids pTLL003 and pTLL004 and transformation of *E. coli* TG-1 Dev electro-competent cells

Sequences flanking the SUB 0809 and *hasA* genes were amplified and digested overnight with *Bam*HI or *Kpn*I respectively. Digested products were ligated with quick ligase (NEB) to create constructs of approx. 2 kb which were further amplified using PCR (primers *hasA* or SUB 0809 01 & 04). A precise depiction of the constructs produced is shown in **Figure 6.3**. Constructs were blunt-end polished and ligated into PCR®II-Blunt-TOPO®. Plasmid DNA preparations were obtained from transformed cells, and this DNA, along with pG$^+$host9 plasmid DNA was digested with *Xho*I and *Spe*I (for the SUB 0809 deletion construct) or *Msp*I (for the *hasA* deletion construct). To prevent re-circularisation, *Msp*I digested pG$^+$host9 DNA was treated with alkaline phosphatase. Electrophoresis was used to isolate the digested plasmid and TOPO-derived inserts required; these fragments were excised from the agarose gel and purified using the QIAquick Gel Extraction kit. Subsequently, purified vector and insert were ligated together using T4 DNA ligase at a ratio of 2 to 8 (vector to insert). The resulting ligation mixtures were heat-inactivated at 65 ^{o}C for 10 m, then dialysed using VS membranes prior to being used to transform

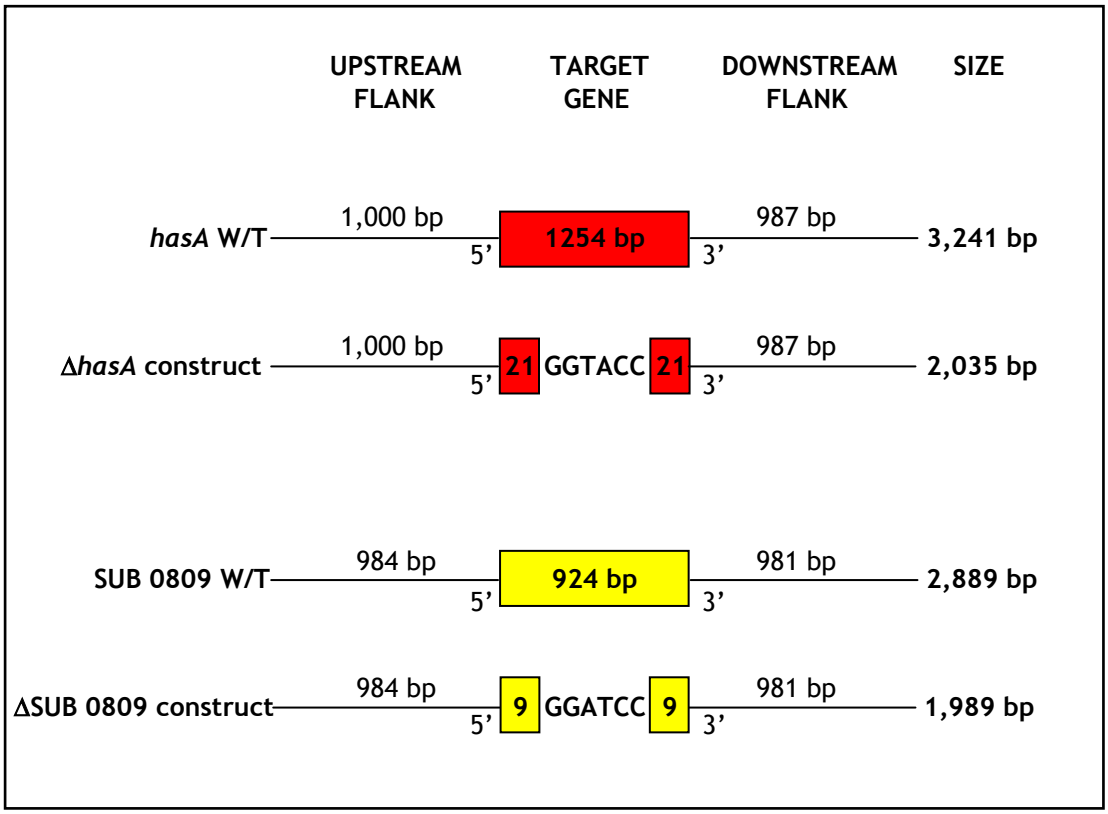


Figure 6.3: Constructs produced for the allele replacement of *S. uberis hasA* and SUB 0809 genes. Deletion constructs are compared to the corresponding wild-type gene region. The size of the upstream and downstream fragments was kept approx. equal to increase the rate of recombination. To ensure the mutation did not shift subsequent genes out of frame, the regions of the target gene downstream of the start and upstream of the stop codons were maintained in multiples of three nucleotides. Changes in the mutant phenotype should thus be attributable to the engineered mutation only. The sequence displayed between the two remaining fragments of the mutated gene, represents the restriction site engineered into the mutant to permit the ligation of the two fragments.

electro-competent *E. coli* TG-1 Dev cells by electroporation. Transformed cells were identified by growth on erythromycin-containing LB agar.

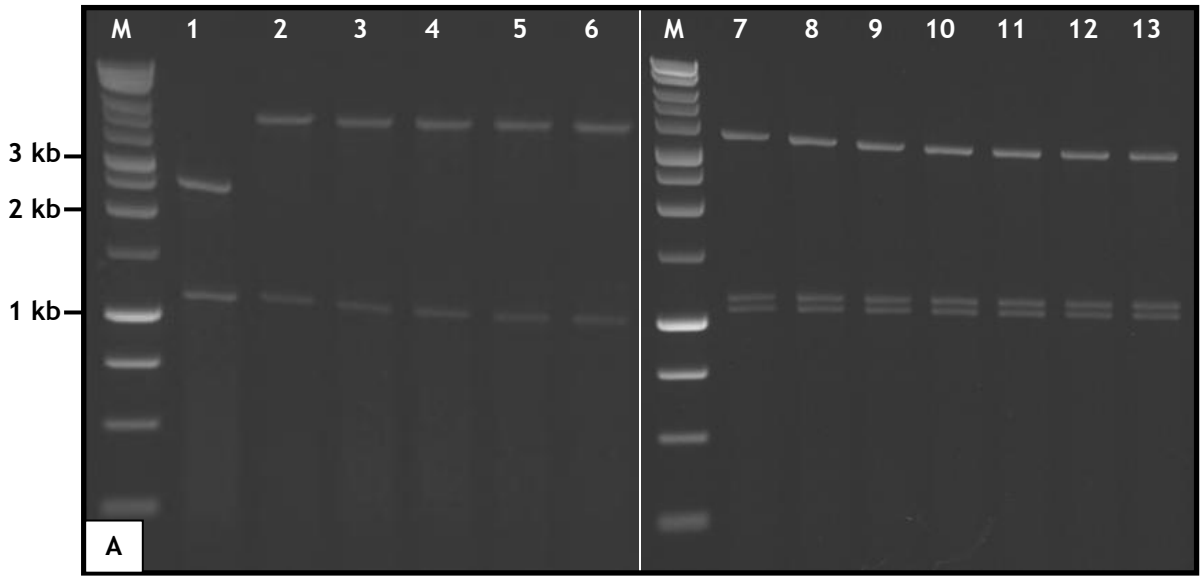
Recombinant plasmids from antibiotic resistant cells were screened by restriction endonuclease digestion and constructs containing the SUB 0809 and the *hasA* deletion were identified and designated pTLL003 and pTLL004 respectively (**Figure 6.4**). Maps of the two novel plasmids are shown in **Figure 6.5**.

Glycerol stocks of *E. coli* cells transformed with plasmids pTLL003 or pTLL004 were prepared. Large-scale plasmid preparations were also conducted using the QIAprep Plasmid Maxi Kit to obtain sufficient plasmid of high quality for subsequent transformation of *S. uberis*. Maxi prep-purified plasmids were quantified and samples digested with *BamHI* to confirm identity. A summary of the mutagenesis workflow is given in **Figure 6.6**.

6.2.5.2 Transformation of *S. uberis* with pTLL003 and pTLL004

Electro-competent *S. uberis* 0140J cells were transformed, as described in **Section 6.2.3** with pTLL003 and pTLL004, the frequencies with which these cells were transformed were 4.1×10^{-5} and 5.2×10^{-6} % respectively. Plasmid DNA, prepared by mini-preps from erythromycin resistant colonies, was screened by restriction endonuclease digestion with *Bam*HI to confirm that the correct plasmid had been introduced into the cells. Transformants containing the desired plasmids were obtained (data not shown) and glycerol stocks were prepared and stored.

Despite failure in earlier experiments, further attempts were made to transform *S. uberis* strains 20569 and T1-20, since both manifested increased biofilm formation in comparison to 0140J. Transformation experiments were conducted, using pTLL004 (or pG^{+}host9 as a control). Plasmid DNA extracted from either *E. coli* TG-1 Dev or *S. uberis* 0140J was used, the later being expected to induce less of a restriction modification effect. In addition, the strains 0140J and T1-60 (which had previously been transformed successfully) were included as positive controls for transformation. Transformations were conducted on two separate occasions. For each experiment, where identified, a single erythromycin-resistant colony was selected and DNA extracted to confirm the presence of the correct plasmid within the cells. As expected, 0140J was transformed at a much greater rate when using the plasmid (pTLL004) derived from *S. uberis* 0140J (6.8×10^{-5} %) rather than that from *E. coli* (1.5×10^{-7} %). Surprisingly, however, there was little difference between the transformation rates obtained for strain T1-60 when either



B	Plasmid	Predicted *Bam*HI digestion fragments (bp)
	pG$^+$host9 (No construct)	2,578, 1,138 and 36
	pTLL003 (ΔSUB 0809)	3,562, 1,138 & 1,060
	pTLL004 (Δ*hasA*)	4,919, 1,138, 142 & 36

Figure 6.4: DNA fragment patterns produced following *Bam*HI digestion of plasmid DNA from transformed *E. coli* cells (A) and the predicted DNA fragment sizes (B). Lane 1 contains digested pG$^+$host9 plasmid DNA, lanes 2 to 6 contain digested pTLL004 (Δ*hasA*) DNA from five distinct erythromycin resistant colonies and lanes 7 to 13 contain digested pTLL003 (ΔSUB 0809) DNA from seven distinct erythromycin resistant colonies.

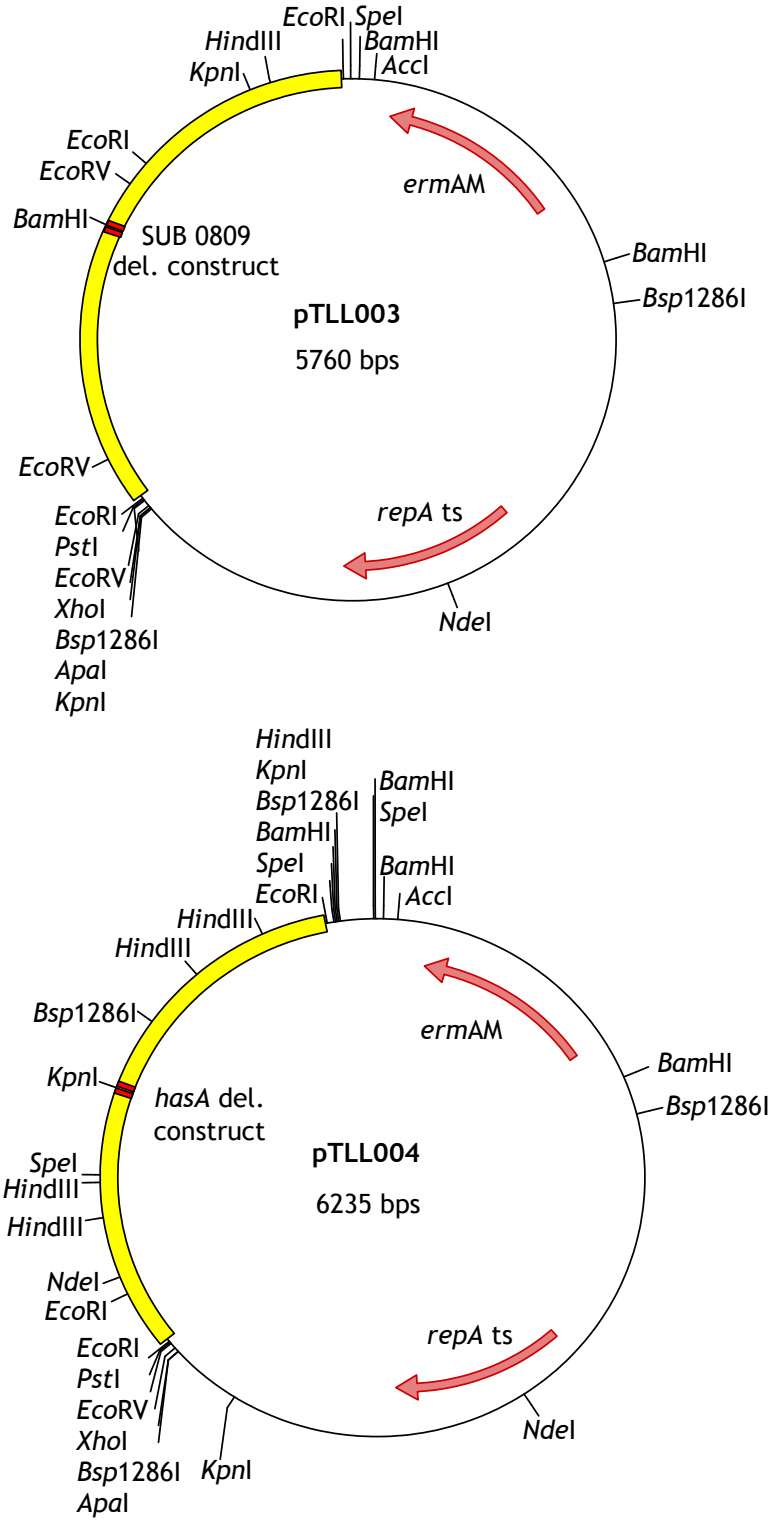


Figure 6.5: Diagram of the constructed plasmids pTLL003 and pTLL004. Plasmid pTLL003 contains the SUB 0809 deletion construct, whilst pTLL004 contains the *hasA* deletion construct. Both constructs were ligated into the vector (plasmid pG^{+}host9).

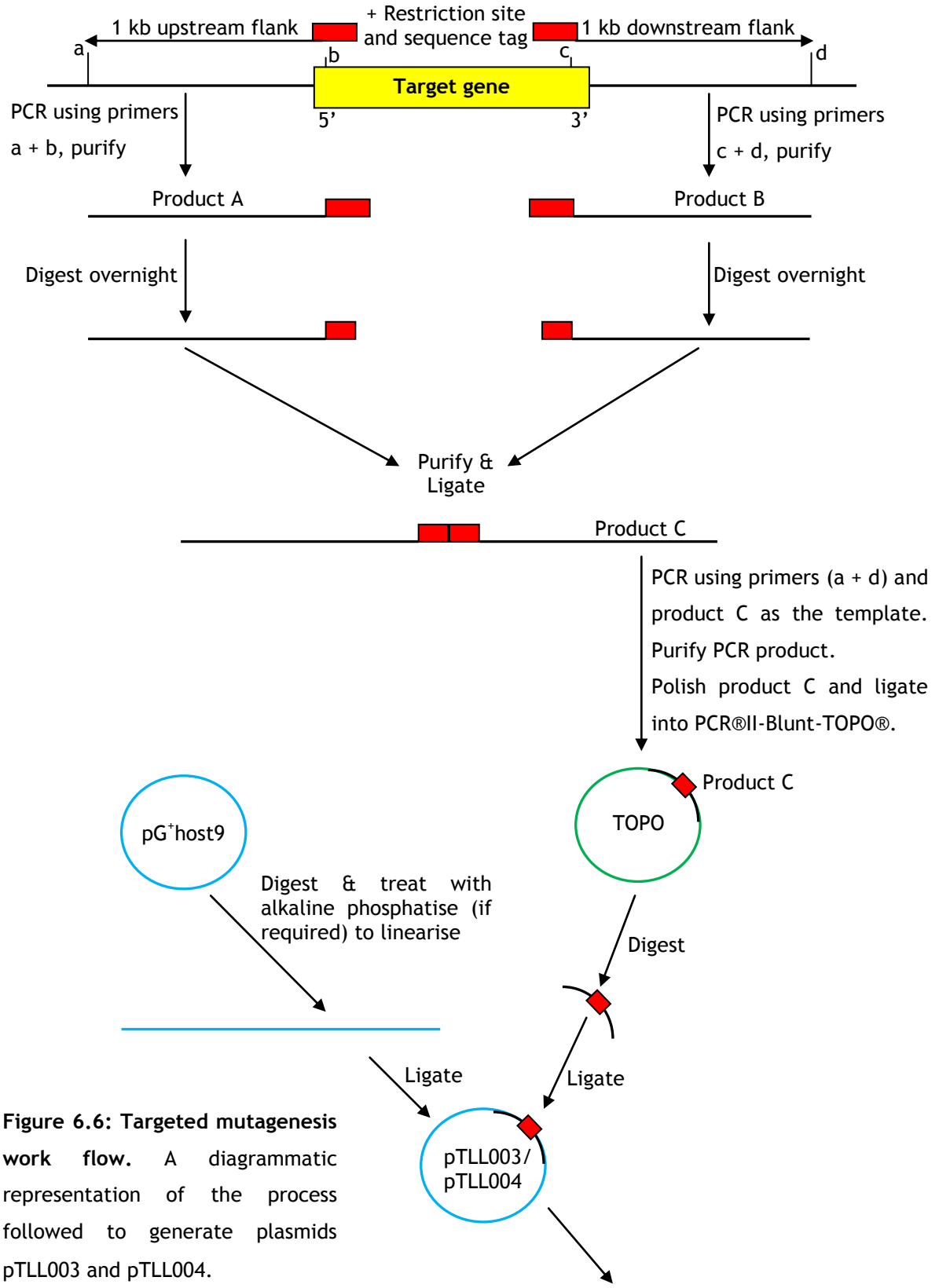


Figure 6.6: Targeted mutagenesis work flow. A diagrammatic representation of the process followed to generate plasmids pTLL003 and pTLL004.

of the pTLL004 plasmid preparations were used (% transformation 2.8×10^{-6} when using plasmid from *E. coli* and 1.9×10^{-6} when using plasmid from *S. uberis*). Again, strain T1-20 could not be transformed with any of the plasmids. In contrast however, strain 20569 was successfully transformed with pG^{+}host9 (derived from *E. coli*) and pTLL004 derived from *S. uberis*, although in each experiment only a single erythromycin resistant colony was obtained (transformation frequency 4.2×10^{-8} %). The DNA banding patterns obtained, following purification and digestion of plasmid DNA, were as expected for all antibiotic resistant colonies tested (data not shown).

6.2.5.3 Construction of defined *S. uberis* mutant strains

Construction of targeted allele-replacement mutants was conducted, as described in **Materials and Methods** using pTLL003 and pTLL004. The frequency with which the plasmids were integrated into the bacterial chromosome, in the first step of the process, was 1.3×10^{-4} and 1.5×10^{-4} % for pTLL003 and pTLL004 respectively. After the second recombination step and the ampicillin enrichment protocol, a total of 21 confirmed erythromycin-sensitive colonies were identified for ΔSUB 0809 and 25 for Δ*hasA*.

Upon excision of the pG^{+}host9-based plasmids from the bacterial chromosome, unless a selectable marker has been engineered into the mutant allele, there is an equal chance that either the wild-type or the mutant genotype will remain (dependent upon whether subsequent homologous recombination events occurred at opposite ends of the target gene (**Figure 6.7**). Determination of whether or not plasmid excision had left the wild-type or mutant alleles within the chromosome, for each putative mutant, was demonstrated by colony PCR amplification of the chromosomal region containing the target genes, using primers SUB 0809 11 and 14b for ΔSUB 0809 and *hasA* 01 and 04 for Δ*hasA*. Three of 10 potential Δ*hasA* mutants, and 11 of 30 potential ΔSUB 0809 mutants were identified where the PCR products were approx. 1,206 bp (Δ*hasA*) or 900 bp (ΔSUB 0809) smaller than the products from the wild-type strain, implying successful allele replacement. Glycerol stocks of these mutants were prepared for storage, gDNA extraction and further confirmation of gene deletions.

6.2.5.4 Confirmation of deletion mutant production

Genomic DNA was extracted from 3 putative *has*A and SUB0809 deletion mutants (identified in **Section 6.2.5.3**), and PCR was utilised to confirm target gene deletion by comparison with the wild-type 0140J strain. Primers were designed which bound out-

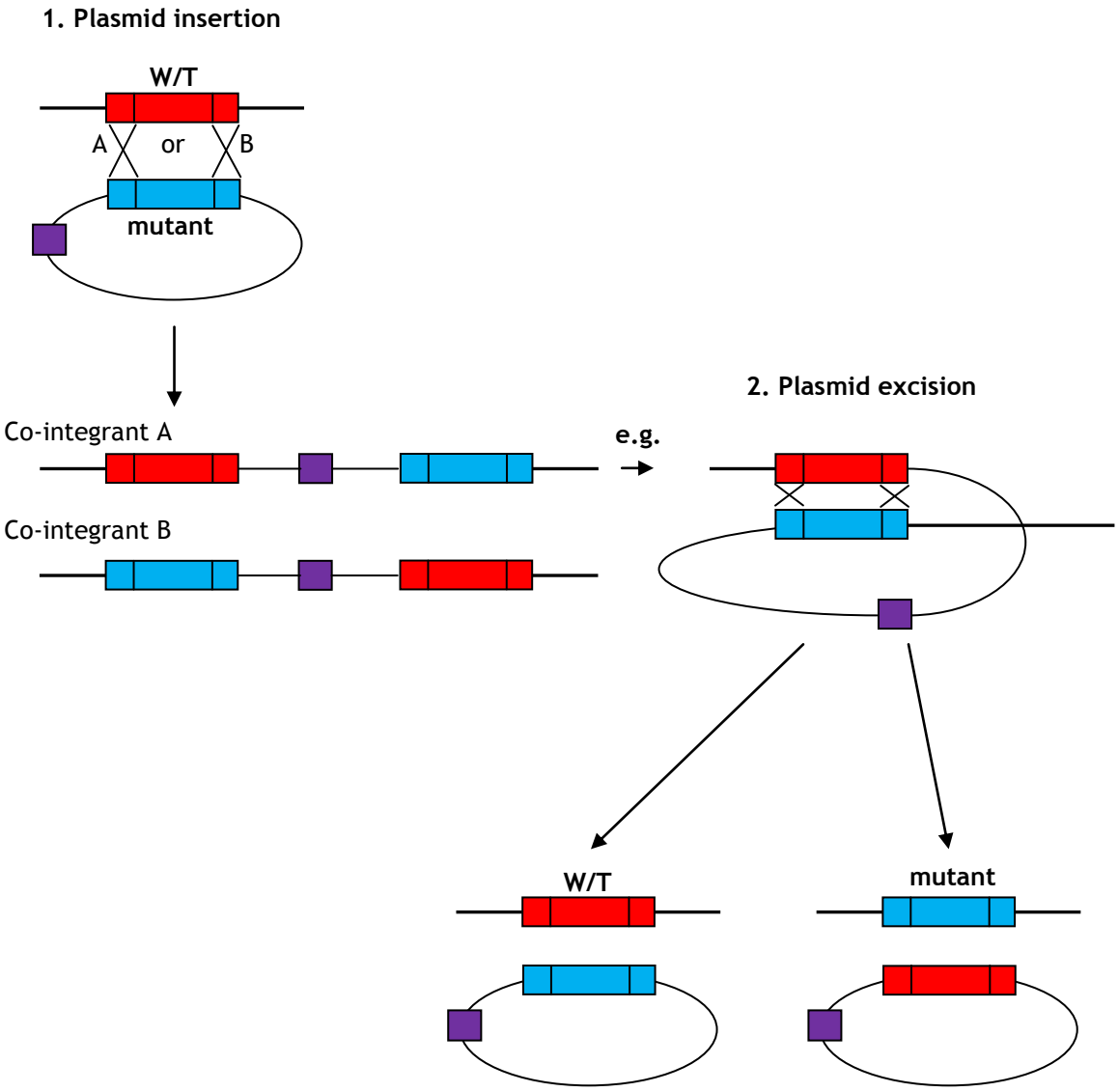


Figure 6.7: Schematic representation of the production of single and double cross over mutants during allele exchange mutagenesis. Firstly (1), the entire plasmid is inserted into the chromosome by recombination between the homologous regions on either side of the target gene. Secondly (2), a further single recombination between the homologous regions of the co-integrant permits the plasmid to be excised from the bacterial chromosome. In this step, the wild-type gene may remain within the bacterial chromosome, or, the mutated gene may be transferred onto the chromosome creating a deletion mutant (Adapted from Walker, 2009).

with the 1 kb flanking regions upstream and downstream from the target genes which were used to create the deletion constructs used for mutagenesis (*hasA* or SUB 0809 Flank 01 and 02). Genomic DNA from all Δ*hasA* and ΔSUB 0809 mutants amplified a PCR product smaller than that obtained from the wild-type gDNA. This further demonstrated that the target genes had been deleted and also, as the primers bound out-with any region present on the plasmids, confirmed that the mutations were present within the chromosome (**Figure 6.8**).

To conclusively demonstrate target gene deletion and to ensure that the deletion had not induced an unwanted shift in the reading frame, amplified PCR products were also sequenced using the additional primers, *hasA* 31 and 32 or SUB 0809 31 and 32. Comparisons of sequences from wild-type and mutants are shown in **Figures 6.9 & 6.10**, demonstrating the deletion of the target genes without the induction of a frame-shift.

The successful deletion of *hasA* was further demonstrated by RT-PCR. Wild-type and mutant strains were cultured in BHI broth until mid-log phase, at which point cells were harvested and mRNA extracted. After RT of mRNA to cDNA the housekeeping gene (*recA*) transcript was successfully amplified in all cases using the primers *recA* F and R. In contrast, no *hasA* transcript was observed in the Δ*hasA* strain using primers *hasA* 21 and 22 (**Figure 6.11**). The absence of contaminating gDNA was confirmed by the inclusion of RT-negative control reactions. Unfortunately, due to technical problems, equivalent confirmation of the absence of a SUB 0809 transcript could not be made during this study.

6.2.5.5 Phenotypic analyses of *S. uberis* mutants

Slime and biofilm assays were used to determine the phenotypic effects, if any, of deleting the *hasA* or SUB 0809 genes on biofilm production. In the first instance, strains were streaked onto CRA plates and incubated at 37 ^{o}C under either aerobic or anaerobic conditions. In both cases, just as with the 0140J wild-type control, it was found that both isogenic mutants still yielded black colonies under both atmospheric test conditions (**Figure 6.12**), implying that exopolysaccharide/slime, is still being produced by the mutant strains.

Biofilm formation was also measured by culturing cells for 48 h in BHI broth or CDMch under aerobic and anaerobic conditions using the microtitre plate assay. Approximate quantification of biofilm mass, and thus extent of biofilm formation, was made by

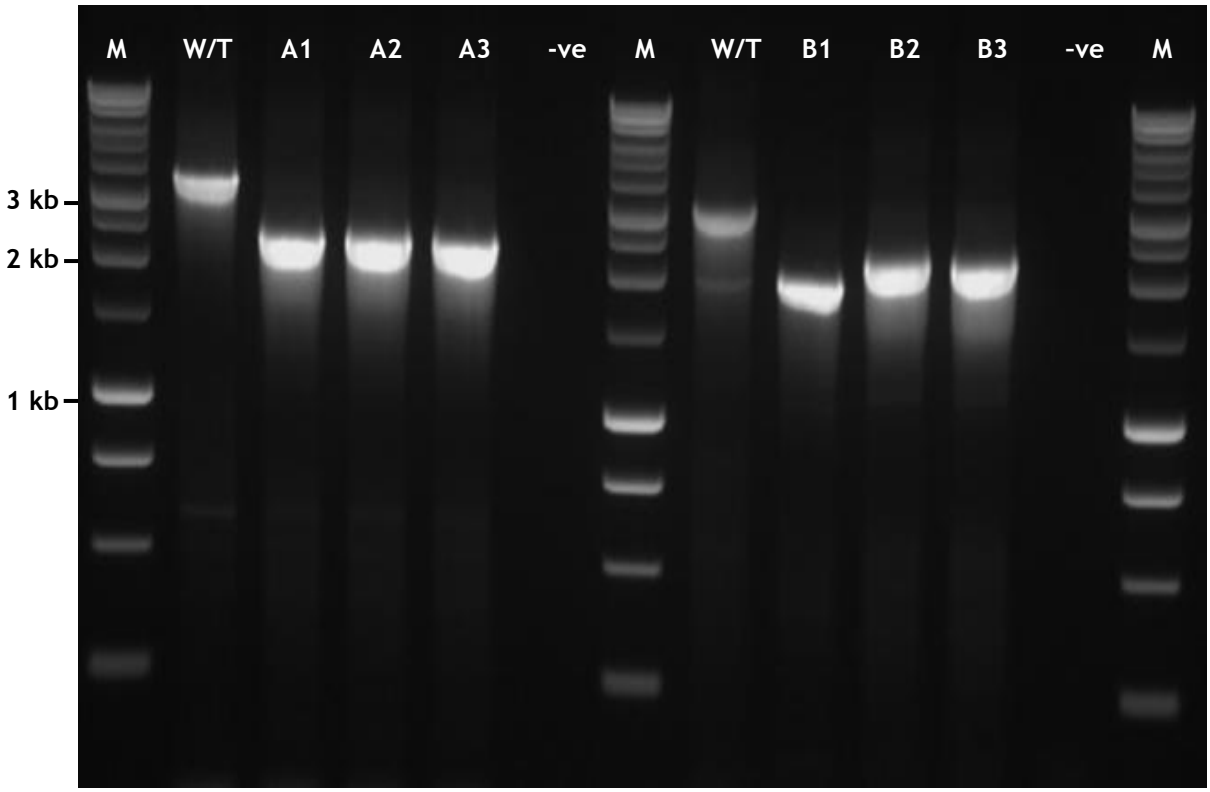


Figure 6.8: Demonstration of target gene deletions in *hasA* (A1-3) and SUB 0809 (B1-3) mutants. Primers (*hasA* or SUB 0809 Flank 01 & 02) targeting regions out-with the regions flanking the target genes which were used for construction of mutant plasmids, were used to demonstrated that the gene deletions lie within the bacterial chromosome and were not simply caused by amplification of a maintained plasmid. The PCR product from mutant B1 was slightly smaller than the size of the predicted product (in which the target gene had been deleted), so mutant B1 was discarded.

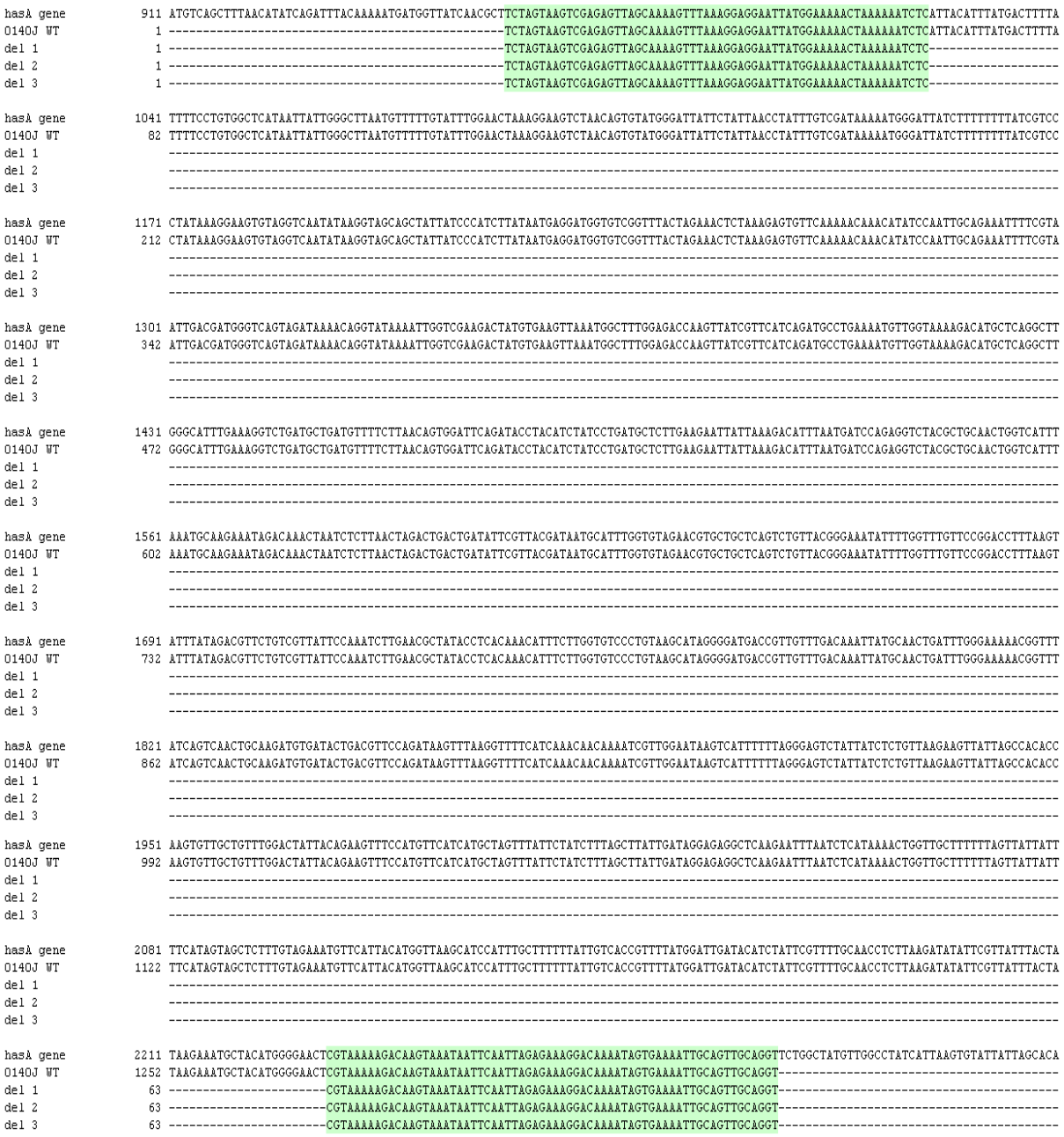


Figure 6.9: Comparison of the nucleotide sequences at the *hasA* locus (*icaA* homologue) derived from deletion mutants, W/T and 0140J genome (AM946015). Edited sequences shown for the W/T and deletion mutants were obtained by aligning both forward and reverse sequences which were obtained on three separate occasions. Edited sequences were aligned, and the obtained image copied from Clone Manager. To simply the alignment, the restriction site introduced into the centre of the deletion constructs was removed (GGT ACC between base pairs 62 & 63)

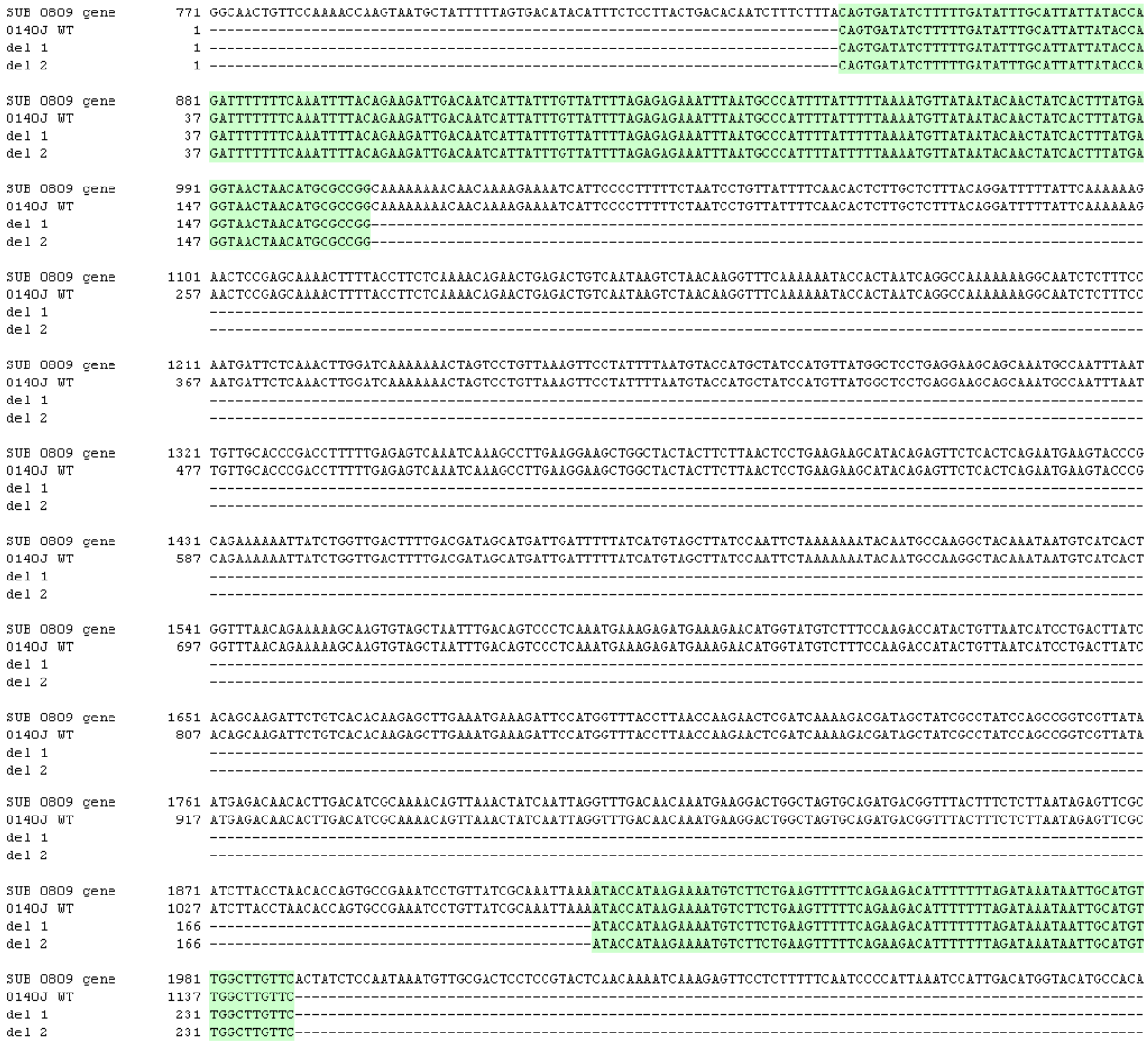


Figure 6.10: Comparison of the nucleotide sequences at the SUB 0809 (*icaB* homologue) locus from deletion mutants, W/T strain and 0140J genome (AM946015). Edited sequences shown for W/T and deletion mutants were obtained by aligning both forward and reverse sequences obtained on three separate occasions. Edited sequences were aligned, and the obtained image copied from Clone Manager. To simply the alignment, the restriction site introduced into the centre of the deletion construct was removed (GGA TCC between base pairs 165 & 166)

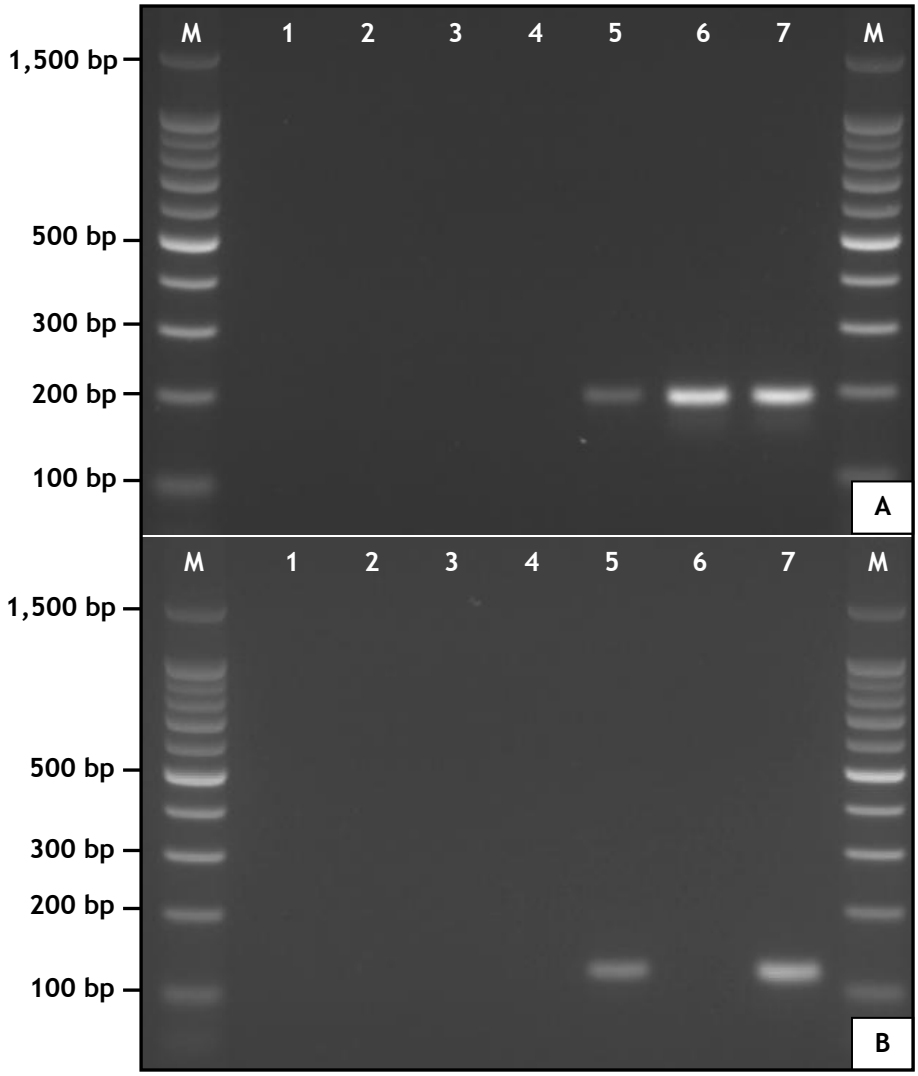


Figure 6.11: Amplification of *recA* (A) and *hasA* (B) transcripts from *S. uberis* 0140J wild-type and mutant cDNA. To obtain cDNA, mRNA was extracted from strains during mid-log phase of growth and subsequent RT yielded equivocal concentrations of cDNA from all strains. Lanes marked M contain 100 bp DNA ladder, lanes marked 1 contain the negative controls. RT negative samples were included as controls for gDNA contamination (Lanes 2-4). These images demonstrate that all strains; W/T (lane 5), Δ*hasA* (lane 6) and ΔSUB 0809 (lane 7) produce transcripts for the housekeeping gene *recA* (A). Alternatively, Δ*hasA* strain does not produce a *hasA* transcript (B). Unfortunately, due to technical problems, during the course of this study it could not similarly be demonstrated that the ΔSUB 0809 mutant did not produce an SUB 0809 transcript.

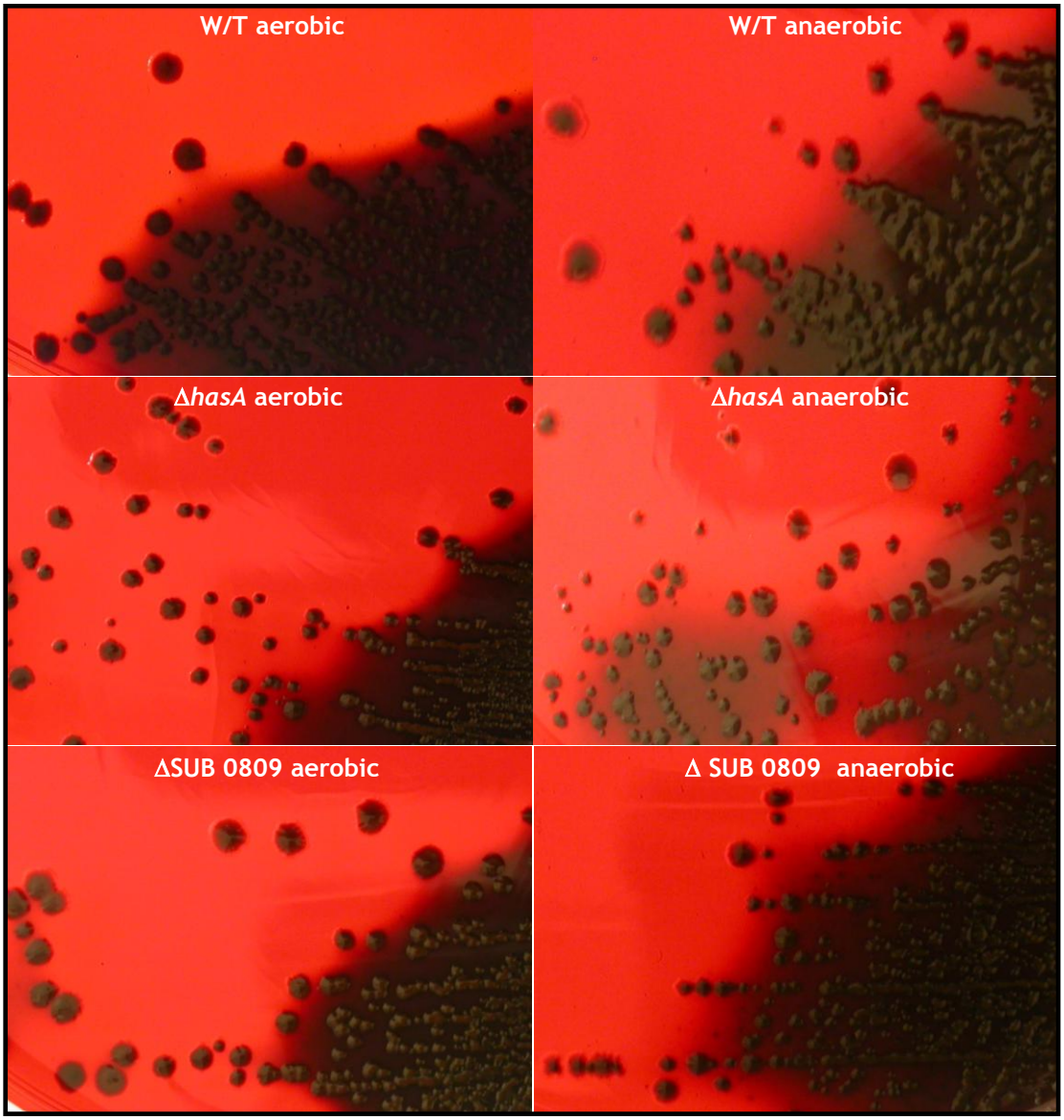


Figure 6.12: Slime production by wild-type *S. uberis* 0140J, Δ*hasA* and ΔSUB0809 mutants under aerobic and anaerobic conditions. No observed change in exopolysaccharide or slime formation was seen between wild-type and mutant strains of *S. uberis* 0140J when cultured on CRA plates.

staining adherent cells with methyl violet, removing stain with ethanol and measuring the intensity of the purple colour by determining the absorbance at 560 nm. Results are presented as the average absorbance values from 6 microtitre plate wells normalised against the average values from two wells containing the corresponding medium only. Unexpectedly, the results of the biofilm assays demonstrated that deletion of the *hasA* gene was not detrimental to biofilm formation, but in-fact dramatically improved biofilm formation under all conditions tested (**Figure 6.13**). In contrast, deletion of the SUB 0809 gene had little effect on biofilm formation under anaerobic growth conditions in BHI broth or CDMch; however, following growth in CDMch in an aerobic atmosphere, biofilm production almost doubled as a result of this mutation.

To determine the effects of gene mutations on *S. uberis* growth characteristics, the mutants and the wild-type strain were cultured simultaneously in BHI broth or CDMch with metal ions using the bioscreen C apparatus. The growth rate was found to be slightly reduced for the ΔSUB 0809 mutants and more noticeably reduced for the Δ*hasA* mutants in comparison to the wild-type strain (**Figure 6.14**). The differences in final absorbance values between wild-type versus isogenic mutant strains were less obvious, although there was a reduction of approx. 10 % in final growth levels achieved by Δ*hasA* mutants following propagation in complex and defined medium compared to the wild-type; whilst there was a growth reduction of approx. 5 % for SUB 0809 mutants in BHI broth compared to the wild-type strain. Growth patterns obtained in BHI broth and CDMch were very similar, except that as expected, growth was slightly accelerated in complex medium and final $OD_{600\ nm}$ values were higher compared to those achieved in defined medium.

6.2.6 Random-insertion mutagenesis using pGh9:IS*S1*

The use of pGh9:IS*S1* for random insertional mutagenesis of *S. uberis* strain 0140J was also trialled during this study, as an alternative, unbiased method for identifying genes involved in biofilm production. Competent *S. uberis* 0140J cells were successfully transformed with the pGh9:IS*S1* plasmid, permitting the preparation of a mutant library (as described in Materials and Methods). Aliquots from the mutant library were diluted and plated onto CRA plates (containing erythromycin) in an attempt to screen for altered phenotypes. Unfortunately, individual mutant colonies lacking the black colony type on CRA could not be identified (data not shown). This, and earlier observations of black colony phenotype on CRA irrespective of the biofilm forming ability of the strain, demonstrate that the CRA assay is insufficient for screening for biofilm mutants. As

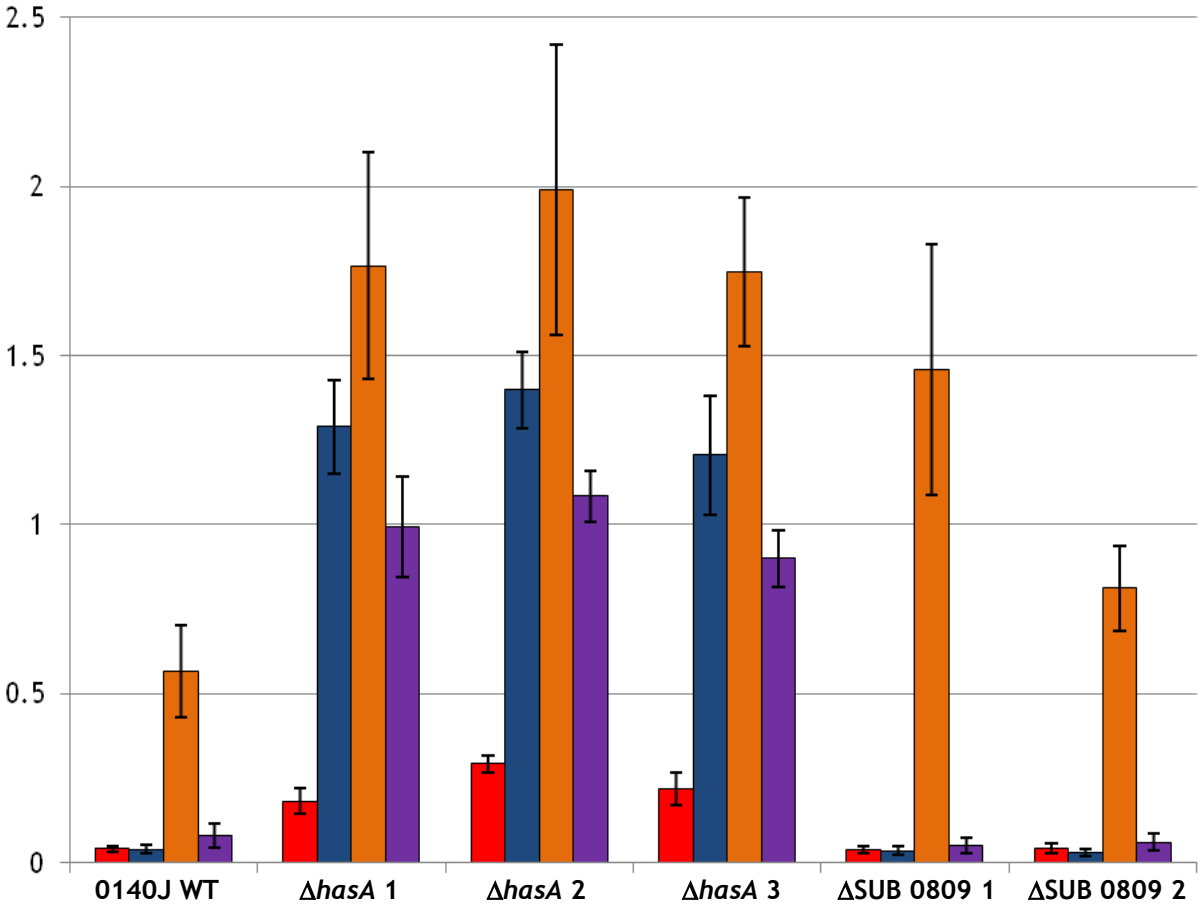


Figure 6.13: Biofilm formation, as determined using microtitre plate assay, by wild-type *S. uberis* and Δ*hasA* and ΔSUB 0809 mutants under different growth conditions. Absorbance at 560 nm, displayed on y axis, is based upon average values obtained from six test wells normalised against the mean absorbance from two corresponding medium only control wells. Red columns represent biofilm formation in BHI broth under aerobic conditions, Blue columns BHI anaerobic, orange columns CDMch aerobic and purple columns CDMch anaerobic. Poor biofilm formation by wild-type strain was evident in all media except defined medium with oxygen. Deletion of the *hasA* gene (*icaA* homologue) resulted in a drastic increase in biofilm formation by the mutant under all conditions whilst the effect of deleting the SUB 0809 gene (*icaB* homologue) was limited to a vast increase following growth in CDMch with oxygen.

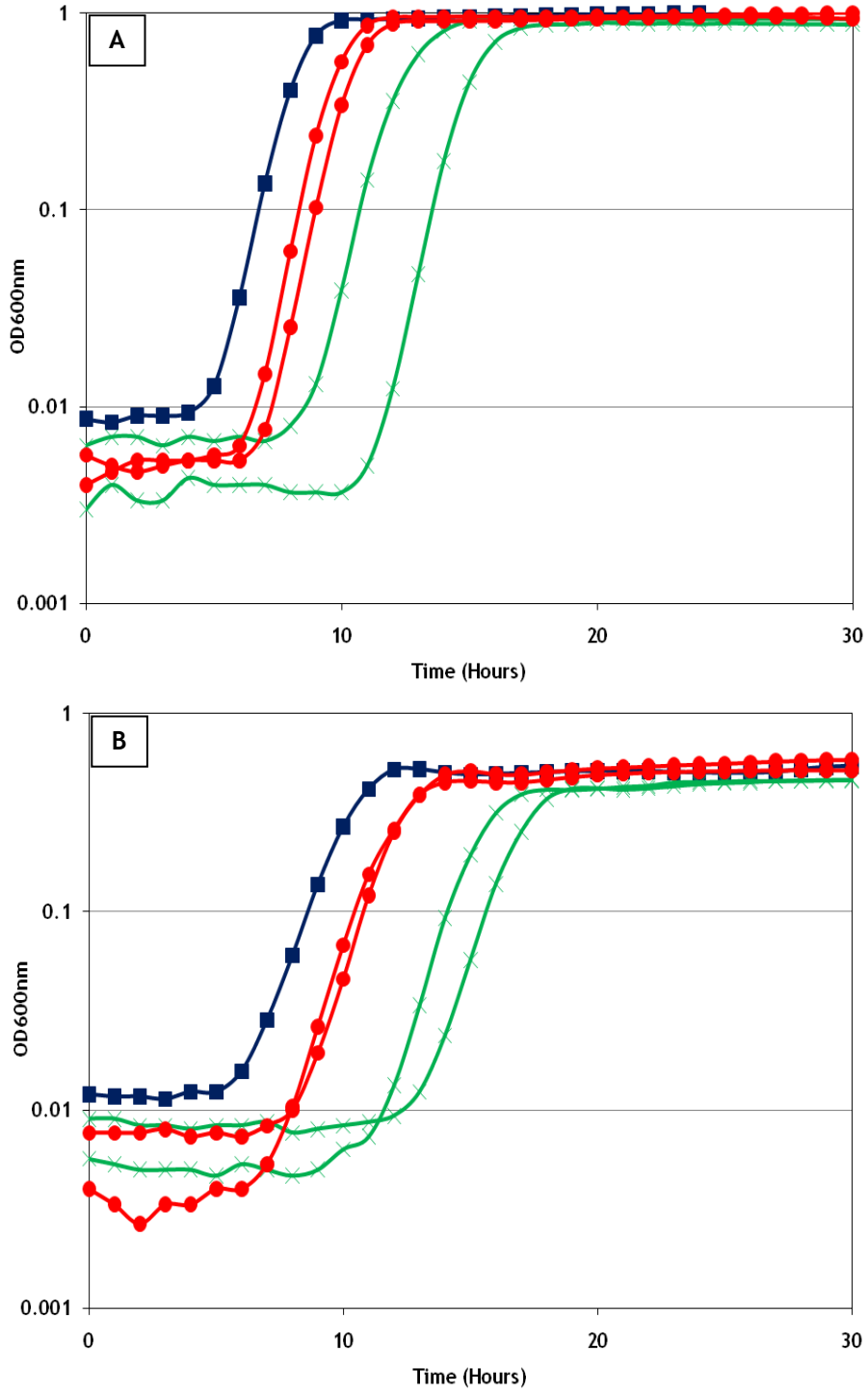


Figure 6.14: Growth characteristics of *S. uberis* 0140J wild-type and mutants strains. Stationary phase cultures prepared in CDMch with metal ions were used to inoculate test media; BHI broth (A) or CDMch with metal ions (B). Whilst final OD values for wild-type 0140J ( ) were almost comparable to those of two Δ*hasA* mutants ( ) and two ΔSUB 0809 mutants ( ), growth rate was clearly affected by both mutations, with Δ*hasA* mutants replicating slower than ΔSUB 0809 mutants. This growth variation was evident in both defined and complex media.

homologues of genes with importance for biofilm formation in staphylococci were identified in the *S. uberis* genome, the development of the site directed/allele replacement mutagenesis protocol was considered a priority and the library was thus not screened any further during the course of this project. By the end of this study an optimised biofilm microtitre plate assay had been developed, it may thus be pertinent for future research to utilise the stored random-insertion mutant library, or to produce a new library using a strong biofilm forming strain, to identify biofilm negative mutants using the microtitre assay. This may permit the identification of completely novel genes which are important for *S. uberis* biofilm development.

6.3 Discussion

The observations presented in **Chapter 5**, that *S. uberis* is able to form biofilms, prompted a search of the *S. uberis* 0140J genome for genes encoding proteins displaying homology to characterised biofilm proteins of other bacteria. At the time when the work presented in this thesis was conducted, the concept of streptococcal biofilms was in its infancy, while in contrast extensive research had been published for *S. aureus* and *S. epidermidis*. For this reason, it was decided to determine whether a system homologous to that encoded by the staphylococcal *ica* operon was also responsible for biofilm production in *S. uberis*. Homologues of the staphylococcal *icaA*, *icaB*, *icaC*, *icaD* and *luxS* genes were identified in *S. uberis* 0140J (using the genome sequence). These genes thus represented potential targets for further study.

In the first instance, the conservation of the *S. uberis* homologues of these genes amongst a collection of *S. uberis* strains was determined. Significantly, the gene from *S. uberis* which shared homology to *icaA* from *S. epidermidis* has previously been reported as *hasA*, encoding a hyaluronan synthase enzyme required for hyaluronic acid capsule formation (Ward *et al.*, 2001). The *hasA* gene was not conserved, however, in all *S. uberis* mastitis strains tested in this study (present in 77 %) and interestingly, all strains lacking *hasA* were able to form biofilms. Although *hasA* positive strains were also capable of forming biofilms, several did not following anaerobic growth in defined medium. The statistical significance of the correlation between absence of *hasA* and biofilm formation was P=0.17 which is not generally considered to denote statistical significance, although the 0.05 cut-off value used to denote statistical significance is also considered by many to be subjective (Rothman and Greenland, 1998). Certainly, analysis of more strains would increase confidence in this observation. Previous reports have also identified *S. uberis* strains which do not harbour *hasA*, whilst a correlation was found between the most dominant multi-locus STs and carriage of *hasA*, which seemed to suggest that this gene may be beneficial for the survival of *S. uberis* in the mammary gland (Coffey *et al.*, 2006; Tomita *et al.*, 2008). This hypothesis was also supported by the observation made during this study, of very high sequence homology at the *hasA* locus in positive *S. uberis* strains. It was reported in **Chapter 4**, however, that highly capsular isolates (as identified by insolubility in acetonitrile) did not belong to the ST 5 CC (dominant in the UK) or the dominant Italian BURST group and biofilm formation by *hasA* negative strains was high (this chapter). Interestingly, deletion of *hasA* has also been shown, in a challenge model, not to be detrimental to the ability of *S. uberis* to infect the mammary gland (Field *et al.*, 2003), but hyaluronic capsule has

been found to be crucial for maturation of *S. pyogenes* biofilms (Cho and Caparon, 2005). Thus, it seems that the role of capsule in biofilm formation is complicated, perhaps inhibiting initial adhesion, but enhancing biofilm maturation, this issue is discussed further below.

PCR screening led to the identification of a gene (SUB 0809) homologous to *icaB* of *S. epidermidis* in over 200 *S. uberis* isolates from persistent, non-persistent, ovine and bovine mastitis infections from the UK and Italy, implying that harbouring this gene may be of value to the host strain; in this respect, disruption of *S. epidermidis icaB* has indeed been shown to prevent production of PIA and subsequent cell aggregation (Heilmann *et al.*, 1996). Interestingly, in this study the sequence heterogeneity at the SUB 0809 locus was extensive, and many of the tested strains had unique sequences. A phylogenetic tree was created to permit visualisation of the relationships between the identified SUB 0809 sequences and biofilm production, allowing the identification of a cluster of closely related sequences derived from strains in which biofilm formation was poor. This may suggest that the sequence, rather than the presence or absence of the gene, could influence the ability of *S. uberis* to produce biofilms. Although not conclusive evidence, it was worth noting that two strains which shared the same ST, but differed in their biofilm phenotype were found to differ in sequence at the SUB 0809 locus only (of all MLST and putative biofilm loci sequenced). In this study, the location of SUB 0809 also appeared to be conserved among a sub-panel of strains. The *ica* operon structure seen in *S. aureus* or *S. epidermidis* (Cramton *et al.*, 1999; Gerke *et al.*, 1998) is not found in the 0140J genome and by demonstration of homology in flanking regions of SUB 0809 it seems likely that the operon structure is also not present in the sub-panel of strains. Whether this is also the case for additional *S. uberis* isolates remains to be seen.

In this study, only 30 % of the *S. epidermidis* IcaD sequence shared homology with any translated region from the *S. uberis* 0140J genome, this being the protein encoded by the ORF designated SUB 0701. Even then, this low coverage was split between two separate areas of the SUB 0701 product. The *S. uberis icaD* gene homologue was also absent from 77 % of additional *S. uberis* strains analysed. Previous research has demonstrated that while IcaD increases the N-acetyl-glucosaminyltransferase activity of IcaA, this enzyme is still capable of functioning in the absence of IcaD (Gerke *et al.*, 1998). Furthermore, no transposon mutants of *S. epidermidis* with reduced or diminished biofilm forming ability were identified in which the *icaD* gene had been disrupted (Mack *et al.*, 2000). It seems unlikely therefore that the product of the *icaD*

gene has an important role in the production of biofilms. In the work presented in this chapter it was observed, however, that most of the *S. uberis* strains from which the *icaD* homologue was amplified (5 of 6) were poor biofilm producers, and whilst insufficient numbers were tested to make any meaningful conclusions, it may instead be debated whether the loss of SUB 0701 from *S. uberis* has in-fact led to increased biofilm forming capacity in these strains (P=0.12). Taken together, these results support the hypothesis that IcaD is not always essential for biofilm formation, and indeed a further hypothesis is raised as to whether the loss of this gene actually increases biofilm capacity, similar to observations with *hasA* (it should be noted that all *hasA* negative strains analysed in the work presented here, also did not harbour SUB 0701). Previously, just 4 % of clinical *Staphylococcus haemolyticus* isolates were found to produce an *icaD* amplicon (as part of the *icaRADBC* operon) and it was later demonstrated that biofilm formation by *S. haemolyticus* utilised a matrix of proteins and DNA, and not PIA, suggesting the molecular basis of biofilm formation was distinct from *S. epidermidis* (Fredheim *et al.*, 2009). The data presented in this chapter suggests that *S. uberis* biofilm formation also occurs in a manner distinct to that reported for many *Staphylococcus* spp., and for that reason further analysis of SUB 0701 is warranted in order to fully determine whether or not it plays a role in *S. uberis* biofilm formation. However, due to constraints of time, no further study of this gene was conducted during this study.

Homology between the *luxS* genes of *S. uberis* 0140J, *S. aureus* and *S. epidermidis* was evident, however, not only does *S. uberis* share considerably higher homology to the *luxS* gene of *S. pyogenes* (91 % identity), but the entire published *S. pyogenes luxS* operon (Lyon *et al.*, 2001) would appear to be conserved in *S. uberis* 0140J (data not shown). This is unsurprising when taking into consideration that the CDSs of the *S. uberis* 0140J genome shared the greatest number of orthologous matches with published genomes from pyogenic streptococci, including *S. pyogenes* (Ward *et al.*, 2009). The *luxS* gene was conserved in all *S. uberis* strains tested, and, in almost half of these strains, the sequenced region of the gene was identical. Strains sharing this allele were, however, both strong and poor biofilm producers. The LuxS system has recently been suggested to function as a QS-based regulator of biofilm formation in *S. epidermidis* (Xu *et al.*, 2006). Despite synthesising an auto-inducer (AI-2), the importance of the LuxS system in QS in other bacterial species has, however, been questioned, as genes encoding receptor systems for AI-2 were not identified in most organisms (Rezzonico and Duffy, 2008; Sun *et al.*, 2004b). Furthermore, inactivation of *S. aureus luxS* was shown not to affect virulence associated traits, including biofilm formation, but did

instead induce a growth defect, suggesting a role for this system in metabolism (Doherty *et al.*, 2006). Subsequently, the function of the LuxS system has generally been considered to be restricted to the recycling of methionine in the activated methyl cycle, a role that had already, and has since, been well described for this protein; downstream effects on biofilm production may thus simply result from the disruption of metabolic pathways unrelated to QS (Holmes *et al.*, 2009; Lebeer *et al.*, 2007; Lee *et al.*, 2006; Rezzonico and Duffy, 2008; Winzer *et al.*, 2002).

The SUB 1487 gene (*icaC* homologue) was also conserved in all tested *S. uberis* strains, but again, no allele at this locus, present in more than 1 strain, corresponded simply to strong or poor biofilm production. Transposon mutagenesis of *S. epidermidis* has demonstrated that disruption of this gene alone is sufficient to prevent biofilm formation (Mack *et al.*, 2000) although it has also been demonstrated recently that deletion of *icaC* changed the structure of the subsequent *S. epidermidis* biofilm to a PIA independent, proteinaceous form (Hennig *et al.*, 2007). Unfortunately, for reason of lack of time, during this study it was not demonstrated whether deletion of the SUB 1487 or *luxS* genes impacted upon *S. uberis* biofilm formation, although this would be interesting work for the future.

An alternative biofilm associated protein, Bap, appears to correlate very well with biofilm formation, and Bap homologues have been found in many of the genomes of staphylococci that form biofilms (Latasa *et al.*, 2006). No such homologue was identified in the genome of *S. uberis* 0140J, but this strain is a rather poor biofilm former and thus may not possess such a virulence associated gene. A search of newly sequenced *S. uberis* genomes might identify such a homologue. Alternatively, in *S. uberis* a genetically distinct protein may serve a similar function.

Despite previous reports of successful transformations of *S. uberis* (Moshynskyy *et al.*, 2003; Smith *et al.*, 2002), in the work described here, considerable optimisation and trouble-shooting was required before *S. uberis* 0140J could be transformed with the plasmid pG^{+}host9. Subsequently, transformation of additional *S. uberis* strains had variable success. Four of thirteen strains tested were not transformed (on one occasion), and transformation efficiency varied from 5×10^{-5} to 9×10^{-3} %. In two further attempts, transformation of one of the previously non-transformable strains was permitted, but an additional strain remained un-transformable, even when the plasmid used for transformation had been prepared from *S. uberis* 0140J in an attempt to counter-act any restriction-modification effects. Whilst restriction-modification systems

of *S. uberis* have yet to be described, two ORFs have been identified from the 0140J genome sequence (Ward *et al.*, 2009) as encoding Type 1 restriction-modification system R and M proteins (SUB 0282 and SUB 0279 respectively). By the end of the optimisation process, conducted during this project, it was possible, however, to transform the majority of *S. uberis* isolates tested with pG^{+}host9. Lack of transformability is the one step which has prevented other research groups from performing routine targeted allele-replacement mutagenesis procedures. Certainly, the generation of 2 distinct allele-replacement mutants in the work described here is the most extensive mutagenesis described in *S. uberis* to-date. The process is now (more-or-less) routine within our laboratory, demonstrating the future opportunities available for analysing the effects of gene deletions in strains which may be more relevant than 0140J (*e.g.* strains which are more frequently isolated from mastitis-causing infections or those which are stronger biofilm formers).

The deletion constructs created during this thesis work were engineered in such a way that the majority of the *hasA* or SUB 0809 gene regions were deleted, in-frame, so as not to introduce polar effects on the transcription of downstream genes. The allele replacement methodology employed to create the *hasA* and SUB 0809-deficient mutants should offer an excellent means of further analysis of the function of these genes. In addition, the use of the pGh9:IS*S1* random insertion library generated could also be put to more extensive use. It is interesting to note that, although a clear (inverse) association with biofilm formation was shown for *hasA* and SUB 0809, the CRA assay failed to show any apparent difference between the mutants and the wild-type parent. Furthermore, out of thousands of colonies, screening of the pGh9:IS*S1* library using the CRA assay also failed to identify, even a single mutant, deficient in Congo Red binding. It is therefore interesting to note that reports questioning the accuracy of this method have been made by others, on the grounds that the results of Congo Red assays often bear no correlation to biofilm measurements made by adhesion to plastic surfaces (Croes *et al.*, 2009; Knobloch *et al.*, 2002). It is therefore entirely possible that, at least in the case of *S. uberis*, Congo Red is being bound not by PIA, but by some as yet undetermined *S. uberis* molecule which has no association with biofilm formation. While this would bring into question the Congo Red assay results presented in this chapter and in **Chapter 5**, the additional assays included in this work have clearly demonstrated biofilm formation by *S. uberis*. Consequently, more definitive methods exist by which such a library could be screened. For example, analysis of individual mutants in microtitre plate-based biofilm assays could be an excellent means of identifying deficient mutants. Similar work has been conducted with *S. gordonii* using a transposon

random-insertion library, and of the genes which were identified as being essential for biofilm formation, the functions of only half were already known (Loo *et al.*, 2000). For the purposes of this study, however, at the point that the Congo Red assay was brought into question, insufficient time remained to undertake the creation of the initial mutant bank required for downstream screening. This would, however, be an interesting area to pursue further in our laboratory.

The microtitre plate assay, which has been widely used by many different research groups for the high throughput analysis of attachment and early biofilm formation (Kristich *et al.*, 2008; McKenney *et al.*, 1998; O' Toole and Kolter, 1998), was thus also used to compare wild-type and mutant *S. uberis* 0140J strains. Using this assay, it was identified that deletion of *hasA*, the *S. epidermidis icaA* homologue, resulted in vastly increased biofilm formation under all growth conditions analysed. Interestingly, it was also previously found, that 5 of 6 strains in the sub-panel which did not amplify *hasA*, all displayed strong biofilm phenotypes following microtitre plate assay (**Chapter 5**). Furthermore, it has also been shown that bacterial capsule hinders biofilm formation by *S. pneumoniae* (Moscoso *et al.*, 2006), *S. suis* (Bonifait *et al.*, 2010) and *S. pyogenes* (Cho and Caparon, 2005), as measured by microtitre plate assay. Experiments conducted in a flow chamber, demonstrated, however, that whilst initial surface binding was observed in the absence of *hasA*, the product of this gene was required for subsequent *S. pyogenes* biofilm maturation (Cho and Caparon, 2005). It seems likely therefore, that deletion of *hasA*, and thus the removal of capsule, increases the initial attachment of bacteria to surfaces, perhaps by exposing more adhesion proteins. It has similarly been shown previously that hyaluronate capsule decreases attachment of *S. pyogenes* to mouse macrophages (Whitnack *et al.*, 1981). The capsule may be needed, however, for continued biofilm propagation, being required, for example, for the creation of the extra-cellular matrix to surround and join bacteria within the biofilm, offering increased protection. To determine if *hasA* is required for the propagation of *S. uberis* biofilms, further work, and more specialised equipment is required.

Deletion of *hasA*, which increases the susceptibility of bacteria to host defences, did not prevent the bacteria from infecting the bovine mammary gland (Field *et al.*, 2003). Recently it was demonstrated that carriage of the *ica* operon was detrimental to survival of *S. epidermidis* on the skin (Rogers *et al.*, 2008), yet it has been repeatedly shown that *ica* genes are required for biofilm formation and that carriage is highly correlated with strains derived from medical device related infections (Heilmann *et al.*, 1996; Li *et al.*, 2005; Ziebuhr *et al.*, 1997). Whether a similar situation occurs in *S.*

uberis has yet to be discovered, either way, it seems likely that capsule expression/loss of capsule may be of great benefit for the colonisation of particular niches. Reports of the ubiquitous isolation of *S. uberis* from the skin of cattle (Cullen, 1966), which it has been claimed is the primary reservoir of the bacteria, may well support this hypothesis. It could also be argued, however, that as *S. uberis* also colonises the bovine gut, bacterial isolation from all areas of the cow is merely a result of faecal contamination of the entire bovine environment. Biofilm formation has been suggested as a mechanism utilised by the enteric pathogen *Campylobacter jejuni* to interact (*in vitro*) with human intestinal tissue (Haddock *et al.*, 2010). Furthermore, biofilm formation by ST 474 strains (a dominant poultry and human-associated type) was high and interestingly, mixed population biofilms with additional bacteria associated with the poultry environment was also demonstrated, suggesting the possibilities for inter and intra-species interactions (and thus genetic exchange) within this environment, which could offer considerable benefits to these bacteria (Teh *et al.*, 2010). Within the dairy environment a similar situation may occur with *S. uberis* (and other mastitis pathogens).

An unexpected growth observation was noted for Δ*hasA* and ΔSUB 0809 mutants compared to the wild-type strain; the growth rate of mutants was slower than that of the wild-type strain, this being particularly noticeable for the Δ*hasA* mutant. As capsule is not essential for *in vitro* or *in vivo* growth of *S. uberis* (Almeida and Oliver, 1993b; Field *et al.*, 2003), deletion of this gene should not impede bacterial growth; indeed, reducing the metabolic burden on the cell through loss of a non-essential gene would normally be expected to improve growth rate slightly. Furthermore, deletion of *S. pneumoniae* capsule gene was found to improve bacterial growth rate (Pearce *et al.*, 2002). It seems that *S. uberis* capsule expression thus may offer some additional benefit to the cell, even in complex medium, that has yet to be defined. As discussed in **Chapter 5**, slower growth rate is also associated with increased biofilm formation (Donlan and Costerton, 2002), thus it is possible that the improved biofilm formation displayed by capsular and SUB 0809 deletion mutants may at least in part be attributable to this reduction in growth rate.

Deletion of SUB 0809 (*icaB* homologue) had the effect of doubling biofilm formation in the mutant compared to the wild-type strain following aerobic growth in defined medium only. Deletion of SUB 0809 had no apparent effect on biofilm production during growth under all additional growth conditions tested. Despite observations that IcaB, a cell surface protein, is essential for PIA modification and biofilm formation (Vuong *et al.*, 2004), it has also been implied that, due to an absence of biofilm negative *S.*

epidermidis mutants in which the transposon was inserted within the *icaB* gene, that *icaB* is not essential for biofilm formation (Mack *et al.*, 2000). Furthermore, *icaB* was previously, contrastingly, shown not to be necessary for *in vitro* biosynthesis of the PIA sugar chain (Gerke *et al.*, 1998). As the precise role of IcaB in biofilm formation remains undetermined it is difficult at this stage to speculate as to why deletion of SUB 0809 improves *S. uberis* biofilm formation, and specifically why this observation is only seen when cells are grown aerobically in defined medium. Further analysis of this mutant, is however, certainly warranted.

The *ica* locus has been shown on many occasions to be essential for biofilm formation by *S. epidermidis,* and in fact, transfer of this locus to biofilm (and *ica*) negative strains, resulted in the development of strains capable of forming biofilms (Li *et al.*, 2005; Mack *et al.*, 2000; McKenney *et al.*, 1998). It was later demonstrated however, that extracellular polysaccharides are not essential for biofilm formation by *S. aureus* (Toledo-Arana *et al.*, 2005) and an *ica* negative *S. aureus* strain was capable of biofilm formation in the presence of Bap, suggesting that other proteins may compensate for the lack of polysaccharide production (Cucarella *et al.*, 2004). Disruption of the *S. epidermidis icaC* gene was indeed also shown recently, to cause the mutant to switch to the production of a proteinaceous biofilm (Hennig *et al.*, 2007). In the results presented in this chapter, it may thus be the case that deletion of *hasA* and/or SUB 0809 may similarly have stimulated a switch to a different (and apparently more efficient) mechanism of biofilm formation. Biofilm development by *S. haemolyticus* has also been shown to rely more upon proteins and extracellular DNA (Fredheim *et al.*, 2009). These reports demonstrate that *ica* independent biofilm formation may be more common than previously thought, and serve to highlight the fact that our understanding of the mechanisms of formation of, and the significance of biofilms to bacteria in their natural environments is currently still limited. Certainly, as the production of biofilms appears inducible and maintainable by multiple mechanisms in a single organism to ensure the phenotype is conserved, the importance that the phenotype plays in bacterial survival is evident.

In conclusion, the aims of the research reported in this chapter were to explore the mechanisms responsible for *S. uberis* biofilm formation using molecular methods. Unfortunately, the requirement for considerable optimisation and trouble-shooting prevented work from progressing as far as was hoped; however, the existing knowledge regarding biofilm formation by *S. uberis* has still been advanced by this study. It seems that the ability of *S. uberis* to form a biofilm may permit the bacterium to survive more

effectively in the mammary gland niche, with biofilm formation correlating well, albeit not uniquely, with strains from persistent infections, perhaps offering at least a partial explanation as to why chronic infections develop. The successful production of targeted *S. uberis* deletion mutants and an IS*S1* mutant library provides the opportunity for further analysis into the effects of additional genes on *S. uberis* biofilm formation. Although not conducted in this study due to time restraints, it would also be interesting to quantify putative biofilm gene expression under different conditions, for example comparing expression during growth in BHI broth or CDM. Advancement of techniques to detect biofilm formation *in vitro* will also progress future discoveries regarding *S. uberis* biofilm development, propagation and regulation. At a later stage, the effect of gene mutations on bacterial pathogenicity in an animal model would also be required to confirm the hypothesis that biofilm formation aids in the development of chronic *S. uberis* infections. Furthermore, analysis of antibiotic sensitivity would also be beneficial to support this hypothesis; unfortunately, as discussed in **Chapter 5**, such an assay could not be optimised to analyse biofilm cells during the time-frame of this thesis study. The information presented in this chapter, further suggests that the use of traditional antibiotics, targeted at planktonic cells, to treat *S. uberis* mastitis may prove to be futile considering the ability of many *S. uberis* strains to exist in a biofilm. This also further illustrates the complexity of the pathogenicity of *S. uberis*.

Chapter 7: General Discussion

Mastitis represents a significant cost to dairy farmers, and *S. uberis* is one of the most commonly recovered bacteria from animals with clinical signs as well as those without clinical signs but whose milk has an elevated SCC. The excessive use of antibiotics is unfavourable, and has also frequently been demonstrated to be ineffective at treating *in vivo* mastitis infections; there has thus, unsurprisingly, been a drive to develop a vaccine against this important mastitis-causing pathogen. In this respect, whole-cell vaccines, which are simple and relatively cheap to manufacture, appear only to offer protection against re-infection with the homologous strain (Finch *et al.*, 1994; Finch *et al.*, 1997). In contrast, sub-unit vaccines have shown greater promise and hence may be the way forward for inducing cross protection against heterologous *S. uberis* strains and even potentially offering protection against additional closely related species (Bolton *et al.*, 2004; Fontaine *et al.*, 2002; Leigh *et al.*, 1999). However, in order to develop a highly effective vaccine, a greater understanding of the heterogeneity at the genetic (and hence proteomic) level of field isolates, in addition to the different mechanisms by which these bacteria may colonise and persist within the host is required. Consequently, the main focus of the work presented in this thesis was to begin to address these questions. In addition, a further objective was to determine whether any genetic relationships or novel phenotypes could be identified to explain why in some cases, despite the use of antibiotic therapy, some infections persist, whilst in other cases *S. uberis* is successfully cleared from the mammary gland.

A collection of *S. uberis* isolates, derived from UK and Italian cases of bovine and ovine mastitis, were characterised by MLST. In agreement with previous epidemiological investigations (Rato *et al.*, 2008; Tomita *et al.*, 2008; Zadoks *et al.*, 2005a) a weakly clonal (half of the isolates in the collection belonged to a single CC), yet recombinatorial population structure (99 STs identified from 176 unique isolates) was demonstrated from analysis of the *S. uberis* collection presented in this thesis. Most significantly, nearly all of the STs that were isolated from more than one animal within the collection were represented in the dominant CC. Furthermore, the dominant STs identified from the UK isolates of this collection, had mostly been identified in previous analyses of UK collections, where they also pre-dominated in a single CC, the ST 5 CC (Coffey *et al.*, 2006; Pullinger *et al.*, 2007). These closely related strains thus appear to be better adapted for survival in the mammary gland. These observations were similar to those made for *S. agalactiae* where a genetically heterogeneous core population was evident, from which virulent, exclusively host-adapted lineages emerged (Sørensen *et al.*, 2010). This also correlated with the observation made in this study that Italian mastitis isolates from sheep did not share any of the same STs with those from bovine

infections, suggesting that strains well suited for survival in the bovine environment might not be equally adept at colonising sheep. Indeed it was interesting to observe that *pauB* (encoding a broader spectrum plasminogen activator) was significantly associated with ovine mastitis isolates, suggesting that PauB may be an advantageous acquisition for the colonisation of sheep. In general, however, the collection of Italian isolates, perhaps as a result of sampling bias or a smaller collection being analysed, were highly heterogeneous; yet the most predominant Italian bovine STs identified were closely related to the dominant STs from the UK.

Given that the *S. uberis* population was shown to be highly recombinatorial, this impacts upon the ability of MLST and downstream analytical tools, such as BURST, to cluster STs which share allelic homology into accurate CCs (Turner and Feil, 2007). Thus, the BURST groups identified in this thesis research (and indeed those presented previously) may not demonstrate descent from a common ancestor; instead, allelic homology may arise through recombination between unrelated strains. Additionally the identification of unique bacteriophage derived regions in the genomes of three phenotypically, genotypically and geographically distinct strains of *S. uberis*, sequenced as part of this thesis (and the identification by Ward *et al.*, 2009 of 3 discrete phage-derived islands in strain 0140J), demonstrates that horizontal gene transfer via transduction contributes to genetic variation between strains, invalidating phylogenetic inferences and influencing virulence (as reviewed by Wagner and Waldor, 2002). From preliminary analysis of the new genome sequences it was clear, however, that these strains shared considerable sequence homology with the previously sequenced 0140J strain (Ward *et al.*, 2009) although a number of novel genes, absent in 0140J, were identified in the three new strains. The translated products of these genes generally shared greatest homology with *S. agalactiae* proteins and were mostly associated with nutrient metabolism. These observations suggest plasticity of the *S. uberis* genome and the capacity to acquire genes which may enable more efficient survival in a particular niche and would also explain why, as determined during this study (data not shown) some *S. uberis* strains survive better in defined medium (in which nutrients are a limiting factor) than others. Inter and intra species recombination and lateral gene transfer were also shown to be driving the genetic variability evident within the *S. dysgalactiae* subsp. *equi* population (McMillan *et al.*, 2010). Virulence or resistance characteristics may thus be acquired and subsequently spread rapidly amongst the population. Although insufficient time was available to conduct a thorough analysis of the novel *S. uberis* genomes, this resource (along with additional genome sequences which have subsequently been completed) represents an opportunity to definitively

determine differences between isolates from persistent and non-persistent infections in the future, which may contribute to an explanation of why the former resist the action of antibiotics *in vivo* whilst the later do not.

The MLST protocol used in this study, specifically targeted housekeeping genes, since this approach allows the investigation of the evolution of a species over long periods of time (Maiden, 2006). The selection of target loci should be researched meticulously before they are chosen to be suitable for a standardised protocol, as it was demonstrated in this study that there were vast differences in the sequence diversity, selection pressure and carriage of the 'housekeeping' genes utilised in the standardised *S. uberis* MLST protocol (Coffey *et al.*, 2006). It is now known that certain bacterial housekeeping proteins may be transported to the cell surface where they can influence bacterial virulence, for example by binding to host constituents. The genes encoding these proteins will thus not be subject to the same selection pressures as genes encoding proteins with a single strict function. The role of housekeeping genes in bacterial virulence has been the subject of review (Pancholi and Chhatwal, 2003) and highly virulent strains of *S. suis* found to exhibit identical glutamate dehydrogenase (a housekeeping gene) sequences regardless of geographical origin, while sequences from moderately and non-virulent strains were distinct (Kutz and Okwumabua, 2008). Further research is required in this area to determine the extent to which housekeeping genes play a role in the virulence of *S. uberis* and indeed other bacterial pathogens, and may offer an alternative approach to vaccination and diagnostics.

At the time when the *S. uberis* MLST scheme was developed (and also when this study was commenced) no genome sequence was available, and so a full understanding of the location of the genes in the MLST panel within the *S. uberis* genome could not be made. It has only been in the last few years that it has become practical to sequence one (or several) genomes, but with the cost of whole genome sequencing reducing continually, whole genome analyses are likely to supersede MLST as a means of discriminating between phenotypically distinct strains of *S. uberis*. Whole genome sequencing and MLST both require, however, a considerable analytical investment, and alternative typing methods which can quickly and easily discriminate between phenotypically distinct isolates are thus still of considerable value. Consequently, the development of such a technique for characterising *S. uberis* isolates formed another avenue of research discussed in this thesis.

Mass spectrometric methods have been utilised with increasing frequency to discriminate between bacterial species and the technique has been found to be very robust and even as accurate as sequencing (Claydon *et al.*, 1996; Evason *et al.*, 2001; Krishnamurthy and Ross, 1996; Mandrell *et al.*, 2005; Mellmann *et al.*, 2008). Researchers are thus also attempting to use this technique to differentiate between strains of the same species (Williamson *et al.*, 2008). In the work described here, it was possible to differentiate between *S. uberis* strains, but only in small-scale experiments. Unfortunately, when experiments were scaled up, the reproducibility of replicates from the same isolate became lower and it was thus harder to differentiate accurately between different isolates, especially as the fingerprints for different isolates were already very similar. The production of thick, insoluble, hyaluronic acid capsules by many isolates further complicated the procedure and impacted upon the extent to which cells could be disrupted using the two protocols which had initially been found to be the most effective. There was however, some limited correlation between clustering demonstrated by BioTyping and MLST, with several isolates from the same farm sharing similar mass profiles and multi-locus STs. Unfortunately, during this study, it could not be determined if differences existed in the protein profiles of strains from persistent or non-persistent infections. Considerable optimisation of the protocol was conducted during this study; however, further work would still be required to permit definitive characterisation of isolates, and, it is yet to be accurately determined if this simple approach (which could be equally utilised for the analysis of all bacterial species associated with mastitis) represents a cheaper or higher throughput alternative to genomic typing.

As MLST demonstrated that isolates in the dominant CC were associated with both persistent and non-persistent infections, and BioTyping could not be utilised to identify a persistence phenotype, alternative methods were required to seek to identify phenotypes that might explain strain behaviour *in vivo*. Firstly, in order to achieve this, a defined growth medium, representative of the *in vivo* growth environment, was developed and utilised in an attempt to identify (*in vitro*) relevant factors. Significantly, this medium permitted the observation that *S. uberis* was able to directly utilise casein as a source of peptides and amino acids, in contrast to previous reports (Kitt and Leigh, 1997) offering an explanation as to why the plasminogen activator (thought to liberate nutrients from casein via activation of plasmin) has been shown not to be essential for *S. uberis* pathogenesis (Ward *et al.*, 2003). Furthermore, bacterial growth seemed to be permitted in the absence of iron, whilst manganese and magnesium were essential for bacterial proliferation; this result is consistent with a

previous report in which manganese was found to be an essential ion (Smith *et al.*, 2003). Further work is required, however, to characterise the nutrient requirements of *S. uberis* during aerobic and anaerobic growth and the preference of the bacteria for fermentation or respiration. Additionally, it was observed that *S. uberis* might produce a siderophore, although admittedly much more work is required to prove or refute this observation, beyond the preliminary observations made during the work reported in this thesis. With respect to the production of an iron-binding molecule, and the consequent metabolic burden imposed upon the cell, the bacterium would only commit resources towards acquiring such an ion if its acquisition supported an alternative metabolic pathway (respiration) and additional factors such as haem and quinone were also available. Interestingly, there is limited data to suggest that siderophores may also be used for manganese acquisition (Parker *et al.*, 2004). To the author's knowledge this is not something that has been studied in-depth, but it may be possible that organisms, like *S. uberis*, use siderophore-like molecules to acquire manganese in the same way that other organisms acquire iron. Certainly, *S. uberis* appears similar in its preference for manganese to other lactic acid bacteria, including, *Lactobacillus* spp. (Elli *et al.*, 2000) and *S. suis* (Niven *et al.*, 1999). Consequently, although only a hypothesis at this time, it is likely that the assay conditions assessed in the current study were inappropriate to fully determine siderophore activity since iron was included as the substrate in the assay, not manganese. As the bacterium was shown to survive in the absence of iron it seems probable, however, that any siderophore production (to acquire iron) would only be required, as a last resort to improve or sustain bacterial growth when additional nutrients become limited, or upon switching to an alternative metabolic pathway. It is possible also, that if indeed an iron-binding siderophore is produced, it is a genetic remnant that no longer serves a distinct purpose, or alternatively, has been acquired along with other sequences by horizontal gene transfer.

Of the observations made using CDM during this thesis work, of the greatest interest was the fact that, under certain conditions, *S. uberis* strains were able to produce biofilms. Furthermore, the ability to form biofilms clearly varied between strains. Generally speaking, biofilm formation was increased by growth in CDM and under anaerobic conditions, *i.e.* exactly those conditions that would be encountered by the organism *in vivo* within the udder environment. Certainly, these observations are in line with the school of thought that biofilm formation is stimulated in response to conditions which force the bacterium to grow slowly, such as nutrient limitation (Amarasinghe *et al.*, 2009; Cramton *et al.*, 2001; Donlan and Costerton, 2002). Most interestingly, when

grown anaerobically in defined medium, there was a trend for high levels of biofilm production to be related to *in vivo* persistence. Thus, *S. uberis* biofilm formation may facilitate the development of chronic mastitis cases which are refractory to antibiotic therapy, as is the situation with *S. aureus* (Melchior *et al.*, 2006a; Melchior *et al.*, 2009).

Considering that deletion of the *ica* genes, which express, modify and localise PNAG/PIA, prevented subsequent biofilm formation (Cramton *et al.*, 1999; Mack *et al.*, 2000) this adhesin appeared to be essential for the generation and regulation of *S. aureus* and *S. epidermidis* biofilms. Homologues of the *S. epidermidis ica* genes were identified during a search of the unpublished and incompletely annotated *S. uberis* 0140J genome. The *S. uberis icaA* homologue was later identified as *hasA*, the product of which is required for hyaluronic acid capsule production (Ward *et al.*, 2001). Following significant optimisation and trouble-shooting, allele replacement mutagenesis was utilised to create 0140J *hasA* (*icaA* homologue) and SUB 0809 (*icaB* homologue) deletion mutants. The characteristics of the isogenic mutant strains were explored and compared to those of the wild-type parent. *S. uberis* strains which did not harbour *hasA* were all capable of forming biofilms and similarly biofilm formation by Δ*hasA* (and ΔSUB 0809) was significantly increased. Interestingly, it has been demonstrated recently that the *ica* operon is not essential for *S. epidermidis* biofilm formation, as an Δ*icaC* mutant biofilm was proteinacious in composition and not reliant upon PIA (Hennig *et al.*, 2007). Research now suggests that additional components (not just polysaccharides) such as protein and extracellular DNA are also important for biofilm structure (Moscoso *et al.*, 2006; Petersen *et al.*, 2005; Whitchurch *et al.*, 2002). Furthermore, in strains of pathogenic *Staphylococcus* lacking *ica* genes, proteins such as Bap (which is believed to have been laterally transferred amongst many staphylococcal species) have also been shown to compensate for a lack of PIA production, resulting in biofilm formation (Cucarella *et al.*, 2004; Tormo *et al.*, 2005). Whilst no *bap* homologue was determined in the genome sequence of *S. uberis* 0140J, the bacterium may utilise additional proteins with similar abilities to circumvent more established routes of biofilm development. Furthermore, strain 0140J is a relatively poor biofilm producer and so a *bap* homologue might only have been acquired by those *S. uberis* strains with greater biofilm producing abilities. Deletion of *ica* gene homologues in *S. uberis* may thus stimulate biofilm formation by additional routes, unrelated to the production of extracellular polysaccharide. The factors involved, and their regulation are still however to be determined. The optimisation of allele replacement and random mutagenesis protocols for *S. uberis* in this study, and the demonstration of

transformability of multiple strains, presents an opportunity for future work to utilise these methods to determine more about the molecular basis of *S. uberis* biofilm formation by distinct strains. Indeed, deletion of the *ica* gene homologues in different *S. uberis* strains may affect the subsequent biofilm forming ability in a completely distinct manner to strain 0140J.

The results presented in this thesis demonstrate that the hyaluronic capsule inhibited biofilm formation (adhesion to a surface, followed by accumulation of cells) as measured indirectly using the methyl violet microtitre plate adhesion assay. Capsule was also shown to inhibit *S. pyogenes* (Cho and Caparon, 2005) and *S. pneumoniae* (Moscoso *et al.*, 2006) biofilm formation, as determined using a similar assay. Removal of capsule improves exposure of cell surface adhesins, permitting greater attachment to inert surfaces or host cells. Maturation of *S. pyogenes* biofilms (observed using a flow chamber), did, however, at a later stage, require capsule proteins (Cho and Caparon, 2005). This implies that, as has been suggested previously for streptococci in an alternative context, bacteria are able to regulate their capsule production such that lack of capsule supports initial attachment, but capsule formation at a later stage of infection is required to permit survival against host inflammatory factors (Whitnack *et al.*, 1981). Capsule expression by *S. uberis in vivo* has, however, not been detected, even for strains that produced capsule *in vitro* (Prof. J. Leigh, personal communication). This identifies the need for additional equipment to more robustly analyse (*in vitro*) biofilms produced by *S. uberis* as well as the need to observe changes in bacterial gene expression during the course of the biofilm development process.

The following hypothesis could be drawn from the observations made in the work presented here; a population, sharing a genetically similar 'core' genome but in which homologous recombination and DNA acquisition occur freely, are responsible for most mastitis infections (the dominant CC/group). *S. uberis* is considered to be an opportunistic pathogen, which through the acquisition of novel genes (by horizontal transfer) and the ability to utilise multiple metabolic strategies (Ward *et al.*, 2009) is capable of surviving in most areas of the dairy environment. The primary reservoir of the bacterium remains a matter of dispute, but clearly strains which have acquired specific genes, have the opportunity to be better adapted for survival in a particular niche. Capsule expression, for example, may prevent desiccation and promote survival on the skin, whilst loss of capsule may permit prolonged survival in the udder by the stimulation of biofilm formation, which may contribute to the development of chronic infections. Biofilm formation may also explain the observation that persistent *S. uberis*

mastitis isolates display increased genetic diversity (as determined by MLST), as mutation and recombination have been shown to be enhanced in biofilms (Conibear *et al.*, 2009; Hannan *et al.*, 2010; Molin and Tolker-Nielsen, 2003) by up to 1,000-fold. The mechanisms of *S. uberis* biofilm formation remain to be fully determined, but like many other bacteria it seems likely that multiple pathways exist to permit the development of this phenotype.

It has been confirmed that strains sharing an identical 'core' genome (as determined by MLST) may vary widely in their 'accessory' genome which results in divergent phenotypes amongst apparently identical STs. The value of MS as an alternative typing tool remains to be determined, but the technique could also be used to good effect for the identification of mastitis-causing pathogens, and hence allow the use of targeted therapeutics in a timely manner. Despite some limitations in the typing protocols employed in this thesis, the information gained has increased the available knowledge regarding the genetic diversity of *S. uberis* field isolates. Furthermore, the knowledge of *S. uberis* pathogenesis has also been advanced, as, at the time this work was conducted, it had not previously been demonstrated that *S. uberis* has the capacity to produce biofilms. Protocols were also optimised for mutagenesis of this bacterium and an attempt made to identify the genes involved in biofilm formation. Additional research in this area, particularly comparing genomes of biofilm producing strains to non-biofilm strains, as well as the use of (the now optimised) mutagenesis procedures to identify and knock out novel genes involved in biofilm formation, will offer an even greater insight into the pathogenesis of this organism, and permit advances in the development of vaccines or alternative therapeutics against this important mastitis pathogen.

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Appendix 1: Miscellaneous recipes for buffers and solutions

Super Optimal Broth (SOC) Medium

20 g Bacto tryptone, 5 g yeast extract, 20 ml 1 M glucose, 2.5 ml 1 M KCl and 2.5 ml 4 M NaCl. Made up to 1 L with dH_20 and sterilised as required.

15 % SDS PAGE Resolving gel

Into a universal tube the following were added;

2.3 ml dH_20, 5.0 ml 30 % acrylamide mix, 2.5 ml 1.5 M Tris-HCl (pH 8.8) and 0.1 ml 10 % SDS. Just before pouring the following were added:

0.1 ml 10 % APS and 0.004 ml TEMED.

5 % SDS PAGE Stacking gel

The following were mixed in a universal tube, with APS and TEMED being added immediately prior to use:

2.2 ml dH_20, 0.67 ml 30 % acrylamide mix, 1.0 ml 0.5 M Tris-HCl (pH 6.8), 0.04 ml 10 % SDS, 0.04 ml 10% APS and 0.004 ml TEMED

5× denaturing SDS PAGE gel loading buffer

1g SDS, 2 ml 2-mercaptoethanol, 10 mg Bromophenol Blue, 1.5 ml 0.5 M Tris-HCl (pH 6.8), 5 g sucrose and 6.5 ml water.

Coomassie Brilliant Blue Protein Stain

600 mg Coomassie Brilliant Blue R-250, 100 ml glacial acetic acid, 240 ml Methanol and 260 ml dH_20.

Protein De-stain solution

800 ml Methanol, 200 ml Acetic acid and 1,200 ml dH_20.

Appendix 2: *S. uberis thlA* (acetyl-coA acetyltransferase) gene sequences

1) 0140J and 20569
TGCAGCACCATCATTTAGGCCTGAGGCATTTCCTGCTGTTACAGTGCCATCTTTTTCAAAGGCTGGACGAAGTTTAGCAA
GACTTTCCATTGTCGTATCAGGACGAGGATGTCCATCTTTTTCAACAATAGTTACTTGCCCTTTACGTCCTGGAACTTCA
ACTGGAACAATTTCTTGAGCGAAACGACCATTTGATATAGCTTCAGCTGCTTTTTGTTGTGATTCAAAAGCAAATTGATC
TTGTTTTTCACGAGAAATATTGTATTTACGAACAACATTTTCTGCTGTATTTCCCATATGAGCAATTCCTGAATTACGTCC
TGATGCTGAACGGTGCCCTTCTAAGTTGGCATCAATCATTTGGACATCTCCCATACGAAAAGCTTGGTAACGAGCGCTAT
CAGGCAATTGATAAGGAGCGCGTGATAATGATTCTGCACCACCCGCAGCAATAATCTTCTTGTTAGAGTGAAGAAGTTCT
AATGATGCTGATACTGCTGATTGAATACCTGAACCACAAATGCGGT

2) T1-59
TGCAGCACCATCATTTAGGCCTGAGGCATTTCCTGCTGTTACAGT**A**CCATCTTTTTCAAAGGCTGGACGAAGTTTAGCAA
GACTTTCCATTGTCGTATCAGGACGAGGATGTCCATCTTTTTCAACAATAGTTACTTGCCCTTTACGTCCTGGAACTTCA
ACTGGAACAATTTCTTGAGCGAAACGACCATTTGATATAGCTTCAGCTGCTTTTTGTTGTGATTCAAAAGCAAATTGATC
TTGTTTTTT**T**ACGAGAAATATTGTATTTACGAACAACATTTTCTGCTGTATTTCCCATATGAGCAATTCC**A**GAATTACGTCC
TGATGCTGAACGGTGCCCTTCTAAGTTGGCATCAATCATTTGGACATCTCCCATACGAAAAGCTTGGTAACGAGCGCTAT
CAGGCAATTGATAAGGAGCGCGTGATAATGATTCTGCACCACCCGC**C**GCAATAATCTTCTTGTTAGAGTGAAGAAGTTCT
AATGATGCTGATACTGCTGATTGAATACCTGAACCACAAATGCGGT

3) I14
TGCAGCACCATCATTTAGGCCTGAGGCATTTCCTGCTGTTACAGTACCATCTTTTTCAAAGGCTGGACGAAGTTTAGCAA
GACTTTCCATTGTCGTATCAGGACGAGGATGTCCATCTTTTTCAACAATAGTTACTTGCCCTTTACGTCCTGGAACTTCA
ACTGGAACAATTTCTTGAGCGAAACGACCATTTGATATAGCTTCAGCTGCTTTTTGTTGTGATTCAAAAGCAAATTGATC
TTGTTTTTCACGAGAAATATTGTATTTACGAACAACATTTTCTGCTGTATTTCCCATATGAGCAATTCCAGAATTACGTCC
TGATGCCGAACGGTGCCCTTCTAAGTTGGCATCAATCATTTGGACATCTCCCATACGAAAAGCTTGGTAACGAGCGCTAT
CAGGCAATTGATAAGGAGCGCGTGATAATGATTCTGCACCACCCGCAGCAATAATCTTCTTGTTAGAGTGAAGAAGTTCT
AATGATGCTGATACTGCTGATTGAATACCTGAACCACAAATGCGGT

4) I34 and I49
TGCAGCACCATCATTTAGGCCTGAGGCATTTCCTGCTGTTACAGTTCCATCTTTTTCAAAGGCTGGACGAAGTTTAGCAA
GACTTTCCATTGTCGTATCAGGACGAGGATGTCCATCTTTTTCAACAATAGTTACTTGCCCTTTACGTCCTGGAACTTCA
ACTGGAACAATTTCTTGAGCGAAACGACCATTTGATATAGCTTCAGCTGCTTTTTGTTGTGATTCAAAAGCAAATTGATC
TTGTTTTTCACGAGAAATATTGTATTTACGAACAACATTTTCTGCTGTATTTCCCATATGAGCAATTCCAGAATTACGTCC
TGATGCTGAACGGTGCCCTTCTAAGTTGGCATCAATCATTTGGACATCTCCCATACGAAAAGCTTGGTAACGAGCGCTAT
CAGGCAATTGATAAGGAGCGCGTGATAATGATTCTGCACCACCCGCAGCAATAATCTTCTTGTTAGAGTGAAGAAGTTCT
AATGATGCTGATACTGCTGATTGAATACCTGAACCACAAATGCGGT

5) T1-3
TGCAGCACCATCATTTAGGCCTGAGGCATTTCCTGCTGTTACAGTGCCGTCTTTTTCAAAGGCTGGACGAAGTTTAGCAA
GACTTTCCATTGTCGTATCAGGACGAGGATGTCCATCTTTTTCAACAATAGTTACTTGCCCTTTACGTCCTGGAACTTCA
ACTGGAACAATTTCTTGAGCGAAACGACCATTTGACATAGCTTCAGCTGCTTTTTGTTGTGATTCAAAAGCAAATTGATC
TTGTTTTTCACGAGAAATATTGTATTTACGAACAACATTTTCTGCTGTATTTCCCATATGAGCAATTCCAGAATTACGTCC
TGATGCTGAACGGTGCCCTTCTAAGTTGGCATCGATCATTTGGACATCTCCCATACGAAAAGCTTGGTAACGAGCGCTAT
CAGGCAATTGATAAGGAGCGCGTGATAATGATTCTGCACCACCCGCAGCAATAATCTTCTTGTTAGAGTGAAGAAGTTCT
AATGATGCTGATACTGCTGATTGAATACCTGAACCACAAATGCGGT

Appendix 3: *S. uberis pauB* gene sequences

1) I2, I3, I8 & I12
TATTGGAATGTTTGGACTACTGGGAATTCCATACAAAGTAGAGGCTATCACTTCAAAAGAAGTTAATTACCAAGAACCAC
AAGGGACTATGTTAACGATAAATATTACCGGAGAAAGCAAGGAGGGACAGTTGCTCCTTTCTCCAGAATATATGCAATTT
CGCTTAACAGCGGGAGAAGTTATTAATAAAAATGAGTTACTGGATAAAGTGCAACTTGTTATAGACAGTGCTGCTTCTAA
CCAGTTCGAAGTTGTTGATTTCAAACCCGAATCAAAGGTGGAATGGTCCTATTTTGACCGCCACGAATTGCTTGATATCA
CTGAAAGAGGATTTATCGTACCTGATTATTCAATTTATGAAAAGAAACCTTCATTTCTCTTAACAGGCCCTGTTATTATTC
AGAGAAAAAGTCCAACAACAGAGAAACAAACTATTGATAGTAACACCGATGTCATTCTTAGACACAGCGTTTCCTTTATG
AAAAGAAACGGCGAATCAATGAATCTTGTTCCAACAACTACACAGCTATCTTCATTTTCTAGAACTGCAAAACCTGGTCAG
TCAATTCAATCAGAAGAACTCTATCAAGCTGCTCAATCACTTTTTAAGCAAACAAAAGAATATCATGAAGGTTATCGATTA
ATCAAACGGTTGAACACATCGATTTCAGAAAATGAAAAAGCCTATCGCAGTGTTTATCAATTTCAAAACGGAAAACCCTTC
AACTATAGGATTTCACAACTAAGAGAGTTTTCAAAATGGGATTATTCAAAAGCAATTAGAGACGATATCATTGAGAACTA
TTACATTTCCAAAAATGGCGATGACTATTACCCTGAACAAATCAAAATTACGCTTGATTTGACGAATGAAGAAGAAACAA
GTATACGTACTTTAACTTTAGATTTGCCAAAAGAAAACACAATTGAGCATATTAAACACTATCTTAGTGAAGCAGGTCTCA
CTGAAATTGACGCTAAAACAGGAGATCATTATTGGTTTAGTCAGAATGTCAAGCAGGTATCATCCTACCATTTCCAGGCT
AACTATTTCAGCAAAGAAAACTCTGATTTGTTCTACTATAAC

2) I7
TATTGGAATGTTTGGACTACTGGGAATTCCATACAAAGTAGAGGCTATCACTTCAAAAGAAGTTAATTACCAAGAACCAC
AAGGGACTATGTTAACGATAAATATTACCGGAGAAAGCAAGGAGGGACAGTTGCTCCTTTCTCCAGAATATATGCAATTT
CGCTTAACAGCGGGAGAAGTTATTAATAAAAATGAGTTACTGGATAAAGTGCAACTTGTTATAGACAGTGCTGCTTCTAA
CCAGTTCGAAGTTGTTGATTTCAAACCCGAATCAAAGGTGGAATGGTCCTATTTTGACCGCCACGAATTGCTTGATATCA
CTGAAAGAGGATTTATCGTACCTGATTATTCAATTTATGAAAAGAAACCTTCATTTCTCTTAACAGGCCCTGTTATTATTC
AGAGAAAAAGTCCAACAACAGAGAAACAAACTATTGATAGTAACACCGATGTCATTCTTAGACACAGCGTTTCCTTTATG
AAAAGAAACGGCGAATCAATGAATCTTGTTCCAACAACTACACAGCTATCTTCATTTTCTAGAACTGCAAAACCTGGTCAG
TCAATTCAATCAGAAGAACTCTATCAAGCTGCTCAATCACTTTTTAAGCAAACAAAAGAATATCATGAAGGTTATCGATTA
ATCAAACGGTTGAACACATCGATTTCAGAAAATGAAAAAGCCTATCGCAGTGTTTATCAATTTCAAAACGGAAAACCCTTC
AACTATAGGATTTCACAACTAAGAGAGTTTTCAAAATGGGATTATTCAAAATGGGATTATTCAAAAGCAATTAGAGACGA
TATCATTGAGAACTATTACATTTCCAAAAATGGCGATGACTATTACCCTGAACAAATCAAAATTACGCTTGATTTGACGAA
TGAAGAAGAAACAAGTATACGTACTTTAACTTTAGATTTGCCAAAAGAAAACACAATTGAGCATATTAAACACTATCTTAG
TGAAGCAGGTCTCACTGAAATTGACGCTAAAACAGGAGATCATTATTGGTTTAGTCAGAATGTCAAGCAGGTATCATCCT
ACCATTTCCAGGCTAACTATTTCAGCAAAGAAAACTCTGATTTGTTCTACTATAAC

3) I23, I32
TATTGGAATGTTTGGACTACTGGGTATTCCATACAAAGTAGAGGCTATCACTTCAAAAGAAGTTAATTACCAAGAACCAC
AAGGGACTATGTTAACGATAAATATTACCGGAGAAAGCAAGGAGGGACAGTTGCTCCTTTCTCCAGAATATATGCAATTT
CGCTTAACAGCGGGAGAAGTTATTAATAAAAATGACTTACTGGATAAAGTGCAACTTGTTATAGACAGTGCTGCTTCTAA
CCAGTTCGAAGTTGTTGATTTCAAACCCGAATCAAAGGTGGAATGGTCCTATTTTGACCGCCACGAATTGCTTGATATCA
CTGAAAGAGGATTTATCGTACCTGATTATTCAATTTATGAAAAGAAACCTTCATTTCTCTTAACAGGCCCTGTTATTATTC
AGAGAAAAAGTCCAACAACAGAGAAAAAAACTATTGATAGTAACACCGATGTCATTCTTAGACACAGCGTTTCCTTTATG
AAAAGAAACGGCGAATCAATGAATCTTGTTCCAACAACTACACAGCTATCTTCATTTTCTAAAACTGCAAAACCTGGTCAG
TCAATTCAAGCAGAAGAACTCTATCAAGCTGCTCAATCACTTTTTGAGCAAACAAAAGAATATCATGAAGGTTATCAATTA
ATCAAACGGTTGAACACATCGATTTCAGAAAATGAAAAAGCCTATCGCAGTGTTTATCAATTTCAAAACGGAAAACCCTTC
AACTATACGATTTCACAACTAAGAGAGTTTTCAAAATGGGATTATTCACAAGCAATTAGAGACGATATCATTGAGAACTAT
TACATTTCCAAAAATGGCGATGACTATTACCCTGAACAAATCCAAATTACGCTTGATTTGACGAATGAAGAAGAAACAAG
TATACGTACTTTAACTTTAGATTTGCCAAAAGAAAACACAATTGAGCATATTAAACACTATCTTAGTGAAGCAGGTCTCAC
TGAAATTGACGCTAAAACAGGAGATCATTATTGGTTTAGTCAGAATGTCAAGCAGGTATCATCCCACCATTTCCAGGCTA
ACTATTTCAGCAAAGAAAACTCTGATTTGTTCTACTATAAC

4) I29
TATTGGAATGTTTGGACTACTGGGAATTCCATACAAAGTAGAGGCTATCACTTCAAAAGAAGTTAATTACCAAGAACCAC
AAGGGACTATGTTAACGATAAATATTACCGGAGAAAGCAAGGAGGGACAGTTGCTCCTTTCTCCAGAATATATGCAATTT
CGCTTAACAGCGGGAGAAGTTATTAATAAAAATGAGTTACTGGATAAAGTGCAACTTGTTATAGACAGTGCTGCTTCTAA
CCAGTTTGAAGTTGTTGATTTCAAACCCGAATCAAAGGTGGAATGGTCCTATTTTGACCGCCACGAATTGCTTGATATCA
CTGAAAGAGGATTTATCGTACCTGATTATTCAATTTATGAAAAGAAACCTTCATTTCTCTTAACAGGCCCTGTTATTATTC
AGAGAAAAAGTCCAACAACAGAGAAAAAAACTATTGATAGTAACACCGATGTCATTCTTAGACACAGCGTTTCCTTTATG
AAAAGAAACGGCGAATCAATGAATCTTGTTCCAACAACTATACAGCTATCTTCATTTTCTAAAACTGCAAAACCTGGTCAG
TCAATTCAATCAGAAGAACTCTATCAAGCTGCTCAATCACTTTTTGAGCAAACAAAAGAATATCATGAAGGTTATCAATTA
ATCAAACGGTTGAACACATCGATTTCAGAAAATGAAAAAGCCTATCGCAGTGTTTATCAATTTCAAAACGGAAAACCCTTC
AACTATACGATTTCACAACTAAGAGAGTTTTCAAAATGGGATTATTCACAAGCAATTAGAGACGATATCATTGAGAACTAT
TACATTTCCAAAAATGGCGATGACTATTACCCTGAACAAATCCAAATTACGCTTGATTTGACGAATGAAGAAGAAACAAG
TATACGTACTTTAACTTTAGATTTGCCAAAAGAAAACACAATTGAGCATATTAAACACTATCTTAGTGAAGCAGGTCTCAC
TGAAATTGACGCTAAAACAGGAGATCATTATTGGTTTAGTCAGAATGTCAAGCAGGTATCATCCCACCATTTCCAGGCTA
ACTATTTCAGCAAAGAAAACTCTGATTTGTTCTACTATAAC

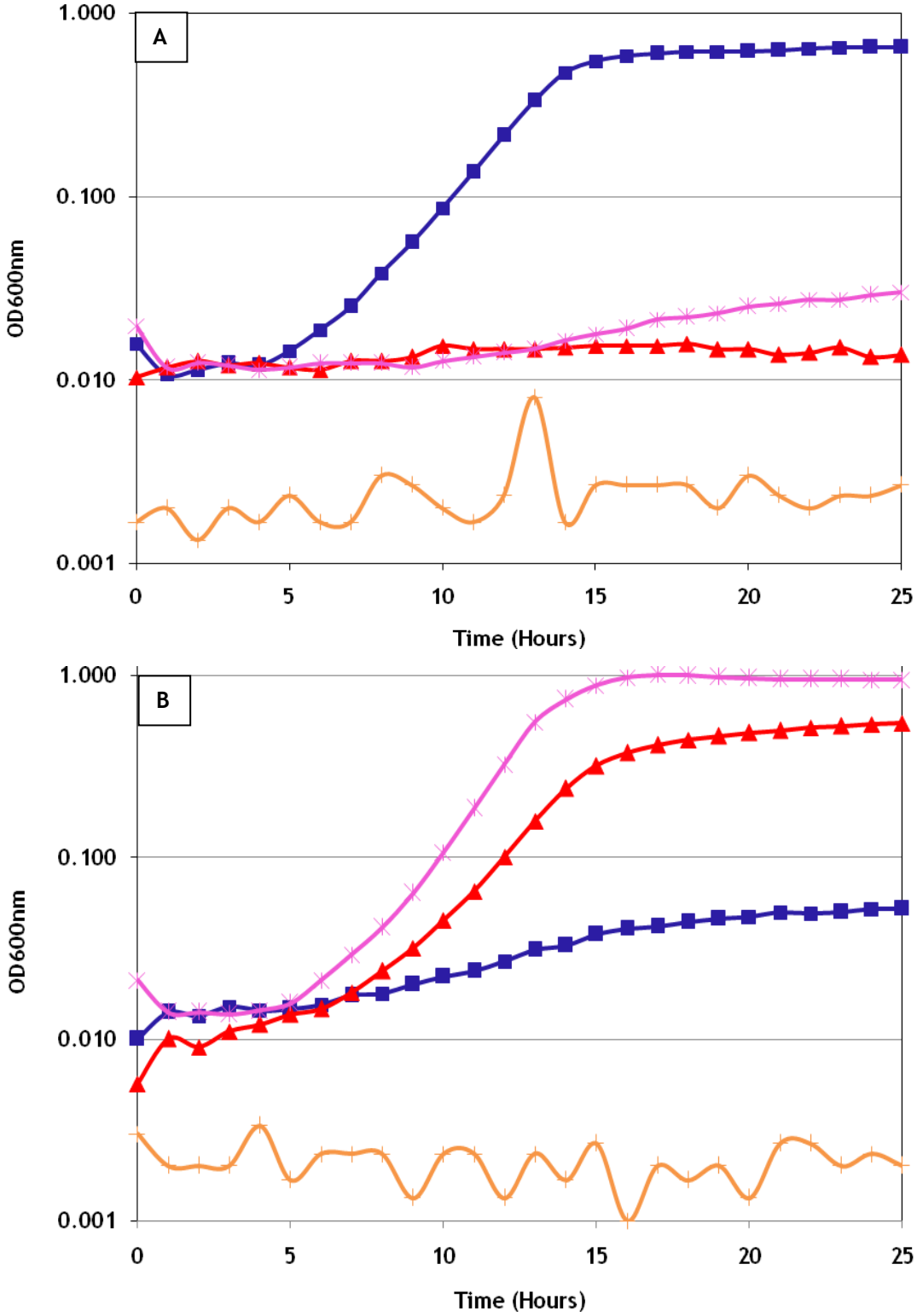
Appendix 4: Reproducibility of the mass/charge values of 20 of the most distinct peaks identified following MS of *S. uberis* 0140J cells obtained from different experiments.

Ribolysis		Acetonitrile	
Average m/z (Da)	SD	Average m/z (Da)	SD
2976.45 *	0.373	2976.75 *	0.582
3192.17	0.388	-	-
3390.98 *	0.725	3391.53 *	0.398
3952.80 *	1.115	3953.00 *	0.219
4069.63 *	0.589	4070.05 *	0.766
4411.48	1.356	-	-
4451.95 *	0.524	4453.07 *	0.186
-	-	4803.08	0.778
-	-	5692.63	1.188
-	-	5733.72	0.471
5957.03 *	0.747	5957.45 *	0.339
6198.62 *	0.979	6199.23 *	0.403
6388.20	0.851	6388.48	0.649
-	-	6721.55	0.689
6786.42 *	0.945	6787.10 *	0.400
-	-	6847.85	0.885
6890.28 *	0.884	6888.80 *	0.970
-	-	6947.82	0.471
7907.40 *	1.071	7908.18 *	0.492
8141.42 *	1.007	8141.73 *	0.771
8203.70	1.121	-	-
8823.38	1.146	-	-
8885.57	1.512	-	-
9489.65 *	1.395	9489.73 *	0.294
9886.67	1.088	-	-
-	-	10138.30	1.375
10874.82	1.339	-	-

Average m/z values from ribolysed cells were calculated from the mass values of peaks identified from two arbitrarily selected replicates of *S. uberis* 0140J analysed on 06th June, 14th June and 04th September 2007. Average m/z values from cells solubilised in acetonitrile were calculated from three replicates selected arbitrarily from experiments on 06th June and 04th September 2007. Standard deviation (SD) values reflect the range of mass values identified for each peak. A SD of <1.5 Da was observed for all peaks.

* Peaks present in spectra obtained using both methods.

Appendix 5: Growth characteristics of *S. uberis* 0140J in published CDM (A) and published CDMch (B).



Stationary phase cultures, prepared in BHI, were used to inoculate CDM on; 05-DEC-06 (—■—), 08-DEC-06 (—▲—), 10-NOV-06 (—✳—) and 19-FEB-08 (—+—).

Appendix 6: Sequences from putative biofilm associated genes

hasA (*icaA* homologue) sequences

1) T2-73, 1:93, 0140J, I6, I26, I38, I40, T1-20, T1-22, T1-36, T1-60, T2-5, T2-10, T2-11, T2-36, T2-53 & T2-67

TCGTCCCTATAAAGGAAGTGTAGGTCAATATAAGGTAGCAGCTATTATCCCATCTTATAATGAGGATGGTGTCGGTTTAC
TAGAAACTCTAAAGAGTGTTCAAAAACAAACATATCCAATTGCAGAAATTTTCGTAATTGACGATGGGTCAGTAGATAAA
ACAGGTATAAAATTGGTCGAAGACTATGTGAAGTTAAATGGCTTTGGAGACCAAGTTATCGTTCATCAGATGCCTGAAAA
TGTTGGTAAAAGACATGCTCAGGCTTGGGCATTTGAAAGGTCTGATGCTGATGTTTTCTTAACAGTGGATTCAGATACC
TACATCTATCCTGATGCTCTTGAAGAATTATTAAAGACATTTAATGATCCAGAGGTCTACGCTGCAACTGGTCATTTAAAT
GCAAGAAATAGACAAACTAATCTCTTAACTAGACTGACTGATATTCGTTACGATAATGCATTTGGTGTAGAACGTGCTGC
TCAGTCTGTTACGGGAAATATTTTGGTTTGTTCCGGACCTTTAAGTATTTATAGACGTTCTGTCGTTATTCCAAATCTTG
AACGCTATACCTCACAAACATTTCTTGGTGTCCCTGTAAGCATAGGGGATGACCGTTGTTTGACAAATTATGCAACTGAT
TTGGGAAAAACGGTTTATCAGTCAACTGCAAGATGTGATACTGACGTTCCAGATAAGTTTAAGGTTTTCATCAAACAACA
AAATCGTTGGAATAAGTCATTTTTTAGGGAGTCTATTATCTCTGTTAAGAAGTTATTAGCCACACCAAGTGTTGCTGTTT
GGACTATTACAGAAGTTTCCATGTTCATCATGCTAGTT

2) I10 & I14

TCGTCCCTATAAAGGAAGTGTAGGTCAATATAAGGTAGCAGCTATTATCCCATCTTATAATGAGGATGGTGTCGGTTTAC
TAGAAACTCTAAAGAGTGTTCAAAAACAAACATATCCAATTGCAGAAATTTTCGTAATTGACGATGGGTCAGTAGATAAA
ACAGGTATAAAATTGGTCGAAGACTATGTGAAGTTAAATGGCTTTGGAGACCAAGTTATCGTTCATCAGATGCCTGAAAA
TGTTGGTAAAAGACATGCTCAGGCTTGGGCATTTGAAAGGTCTGATGCTGATGTTTTCTTAACAGTGGATTCAGATACC
TACATCTATCCTGATGCTCTTGAAGAATTATTAAAGACATTTAATGATCCAGAGGTCTACGCTGCAACTGGTCATTTAAAT
GCAAAAAATAGACAAACTAATCTCTTAACTAGACTGACTGATATTCGTTACGATAATGCATTTGGTGTAGAACGTGCTGC
TCAGTCTGTTACGGGAAATATTTTGGTTTGTTCCGGACCTTTAAGTATTTATAGACGTTCTGTCGTTATTCCAAATCTTG
AACGCTATACCTCACAAACATTTCTTGGTGTCCCTGTAAGCATAGGGGATGACCGTTGTTTGACAAATTATGCAACTGAT
TTGGGAAAAACGGTTTATCAGTCAACTGCAAGATGTGATACTGACGTTCCAGATAAGTTTAAGGTTTTCATCAAACAACA
AAATCGTTGGAATAAGTCATTTTTTAGGGAGTCTATTATCTCTGTTAAGAAGTTATTAGCCACACCAAGTGTTGCTGTTT
GGACTATTACAGAAGTTTCCATGTTCATCATGCTAGTT

3) T2-1

TCGTCCCTATAAAGGAAGTGTAGGTCAATATAAGGTAGCAGCTATTATCCCATCTTATAATGAGGATGGTGTCGGTTTAC
TAGAAACTCTAAAGAGTGTTCAAAAACAAACATATCCAATTGCAGAAATTTTCGTAATTGACGATGGGTCAGTAGATAAA
ACAGGTATAAAATTGGTCGAAGACTATGTGAAGTTAAATGGCTTTGGAGACCAAGTTATCGTTCATCAGATGCCTGAAAA
TGTTGGTAAAAGACATGCTCAGGCTTGGGCATTTGAAAGGTCTGATGCTGATGTTTTCTTAACAGTGGATTCAGATACC
TACATCTATCCTGATGCTCTTGAAGAATTATTAAAGACATTTAGTGATCCAGAGGTCTACGCTGCAACTGGTCATTTAAA
TGCAAGAAATAGACAAACTAATCTCTTAACTAGACTGACTGATATTCGTTACGATAATGCATTTGGTGTAGAACGTGCTG
CTCAGTCTGTTACGGGAAATATTTTGGTTTGTTCCGGACCTTTAAGTATTTATAGACGTTCTGTCGTTATTCCAAATCTT
GAACGCTATACCTCACAAACATTTCTTGGTGTCCCTGTAAGCATAGGGGATGACCGTTGTTTGACAAATTATGCAACTGA
TTTGGGAAAAACGGTTTATCAGTCAACTGCAAGATGTGATACTGACGTTCCAGATAAGTTTAAGGTTTTCATCAAACAAC
AAAATCGTTGGAATAAGTCATTTTTTAGGGAGTCTATTATCTCTGTTAAGAAGTTATTAGCCACACCAAGTGTTGCTGTT
TGGACTATTACAGAAGTTTCCATGTTCATCATGCTAGTT

SUB 0809 (*icaB* homologue) sequences

1) I10 & T1-20

CAAAACTTTTACCTTCTCAAAACAGAACTGAGACTGTCAATAAGTCTAACAAGGTTTCAAAAAATACCACTAATCAGGCCA
AAAAAAGGCAATCTCTTTCCAATGATTCTCAAACTTGGATCAAAAAAACTAGTCCTGTTAAAGTTCCTATTTTAATGTACC
ATGCTATCCATGTTATGGCTCCTGAGGAAGCAGCAAATGCCAATTTAATTGTTGCACCCGACCTTTTTGAGAGTCAAATC
AAAGCCTTGAAGGAAGCTGGCTACTACTTCTTAACTCCTGAAGAAGCATACAGAGTTCTCACTCAGAATGAAGTACCCGC
AGAAAAAATTATCTGGTTGACTTTTGACGATAGCATGATTGATTTTTATCATGTAGCTTATCCAATTCTAAAAAAATACAA
TGCCAAGGCTACAAATAATGTCATCACTGGTTTAACAGAAAAAGCAAGTGTAGCTAATTTGACAGTCCCTCAAATGAAAG
AGATGAAAGAACATGGTATGTCTTTCCAAGACCATACTGTTAATCATCCTGACTTATCACAGCAAGATTCTGTCACACAA
GAGCTTGAAATGAAAGATTCCATGGTTTACCTTAACCAAGAACTTAATCAAAAGACGATAGCTATCGCCTATCCAGCCGG
TCGTTATAATGAGACAACACTTGACATCGCAAAACAGTTAAACTATCAATTAGGTTTGACAACAAATGAAGGACTGGCTA
GTGCAGATGACGGTTTACTTTC

2) T2-10, T2-1, 0140J & T2-11

CAAAACTTTTACCTTCTCAAAACAGAACTGAGACTGTCAATAAGTCTAACAAGGTTTCAAAAAATACCACTAATCAGGCCA
AAAAAAGGCAATCTCTTTCCAATGATTCTCAAACTTGGATCAAAAAAACTAGTCCTGTTAAAGTTCCTATTTTAATGTACC
ATGCTATCCATGTTATGGCTCCTGAGGAAGCAGCAAATGCCAATTTAATTGTTGCACCCGACCTTTTTGAGAGTCAAATC
AAAGCCTTGAAGGAAGCTGGCTACTACTTCTTAACTCCTGAAGAAGCATACAGAGTTCTCACTCAGAATGAAGTACCCGC

AGAAAAAATTATCTGGTTGACTTTTGACGATAGCATGATTGATTTTTATCATGTAGCTTATCCAATTCTAAAAAAATACAA
TGCCAAGGCTACAAATAATGTCATCACTGGTTTAACAGAAAAAGCAAGTGTAGCTAATTTGACAGTCCCTCAAATGAAAG
AGATGAAAGAACATGGTATGTCTTTCCAAGACCATACTGTTAATCATCCTGACTTATCACAGCAAGATTCTGTCACACAA
GAGCTTGAAATGAAAGATTCCATGGTTTACCTTAACCAAGAACTCGATCAAAAGACGATAGCTATCGCCTATCCAGCCGG
TCGTTATAATGAGACAACACTTGACATCGCAAAACAGTTAAACTATCAATTAGGTTTGACAACAAATGAAGGACTGGCTA
GTGCAGATGACGGTTTACTTTC

3) T2-36
CAAAACTTTTACCTTCTCAAAACAGAACTGAGACTGTCAATAAGTCTAACAAGGTTTCAAAAAATACCACTAATCAGGCCA
AAAAAAGGCAATCTCTTTCCAATAATTCTCAAACTTGGATCAAAAAAACTAGTCCTGTTAAAGTTCCTATTTTAATGTACC
ACGCTATCCATGTTATGGCTCCTGAGGAAGCAGCAAATGCCAATTTAATTGTTGCACCCGACCTTTTCGAAATTCAAATC
AAAGCCTTGAAGGAAGCTGGTTACTACTTCCTAACACCTGAAGAAACTTACCGTGTTCTTACTAAAAACGAAGTTCCATC
CGAAAAAATAGTCTGGTTGACTTTTGACGATAGCATGATTGATTTTTATCATGTAGCTTATCCAATTCTAAAAAAATACAA
TGCCAAGGCTACAAATAATGTCATCACTGGTTTAACAGAAAAAGCAAGTGTAGCTAATTTGACAGTCCCTCAAATGAAAG
AGATGAAAGAACATGGTATGTCTTTCCAAGACCATACTGTTAATCATCCTGACTTATCACAGCAAGATTCTGTCACACAA
GAGCTTGAAATGAAAGATTCCATGGTTTACCTTAACCAAGAACTCGATCAAAAGACGATAGCTATCGCCTATCCAGCCGG
TCGTTATAATGAGACAACACTTGACATCGCAAAACAGTTAAACTATCAATTAGGTTTGACAACAAATGAAGGACTGGCTA
GTGCAGATGACGGTTTACTTTC

4) I6 & T1-43
CAAAACTTTTACCTTCTCAAAACAGAACTGAGACTGTCAATAAGTCTAACAAGGTTTCAAAAAATACCACTAATCAGGTCA
AAAAAAGGCAATCTCTTTCCAATGATTCTCAAACTTGGATCAAAAAAACTAGTCCTGTTAAAGTTCCTATTTTAATGTACC
ACGCTATCCATGTTATGGCTCCTGAGGAAGCAGCAAATGCCAATTTAATTGTTGCACCCGACCTTTTCGAAAGTCAAATC
AAAGCCTTGAAGGAAGCTGGTTACTACTTCCTATCACCTGATGAAACTTACCGTGTTCTTACTAAAAACGAAGTTCCATC
CGAAAAAATAGTCTGGTTGACTTTTGACGATAGCATGATTGATTTTTATCATGTAGCTTATCCAATTCTAAAAAAATACAA
TGCCAAGGCTACAAATAATGTCATCACTGGTTTAACAGAAAAAGCAAGTGTGGCTAATTTGACAGTCCCTCAAATGA
AAGAGATGAAAGAACATGGCATGTCCTTCCAAGACCATACTGTTAATCATCCTGACTTATCACAACAAGATTCTGCCACAC
AAGAACTTGAAATGAAAGATTCTATGGTTTACCTTAACCAAGAACTCGATCAAAAGACGATAGCTATTGCCTATCCGGCC
GGTCGTTATAATGAGACAACACTTGATATCGCAAAACAGTTAGACTATCAATTAGGTTTAACAACAAATGAAGGACTAGC
CAGTGCTGATGATGGTTTACTTTC

5) T1-60 & T2-67
CAAAACTTTTACCTTCTCAAACCAGAACTGAGACTGTCAATAAGTCTAACAAGGTTTCAAAAAATACCACTAATCAGGCCA
AAAAAAGGCAATCTCTTTCCAATGATTCTCAAACTTGGATCAAAAAAACTAGTCCTGTTAAAGTTCCTATTTTAATGTACC
ATGCTATCCATGTTATGGCTCCTGAGGAAGCAGCAAATGCCAATTTAATTGTTGCACCCGACCTTTTCGAAAGTCAAATC
AAAGCCTTGAAGGAAGCTGGTTACTACTTCCTAACACCTGAAGAAACTTACCGTGTTCTTACTAAAAACGAAGTTCCATC
TGAAAAAATAGTCTGGTTGACTTTTGACGATAGCATGATTGATTTTTATCATGTAGCTTATCCAATTCTAAAAAAATACAA
TGCCAAGGCTACAAATAATGTCATCACTGGTTTAACAGAAAAAGCAAGTGTGGCTAATTTGACAGTCCCTCAAATGA
AAGAGATGAAAGAACATGGCATGTCCTTCCAAGACCATACTGTTAATCATCCTGACTTATCACAACAAGATTCTGCCACAC
AAGAACTTGAAATGAAAGATTCTATGGTTTACCTTAACCAAGAACTCGATCAAAAGACGATAGCTATTGCCTATCCGGCC
GGTCGTTATAATGAGACAACACTTGATATCGCAAAACAGTTAGACTATCAATTAGGTTTAACAACAAATGAAGGACTAGC
CAGTGCTGATGATGGTTTACTTTC

6) I34
CAAAACTTTTACCTTCTCAAAACAGAACTGAGACTGTCAATAAGTCTAACAAGGTTTCAAAAAATACCACTAATCAGGTCA
AAAAAAGGCAATCTCTTTCCAATGATTCTCAAACTTGGATCAAAAAAACTAGTCCTGTTAAAGTTCCTATTTTAATGTACC
ACGCTATCCATGTTATGGCTCCTGAGGAAGCAGCAAATGCCAATTTAATTGTTGCACCCGACCTTTTCGAAAGTCAAATC
AAAGCCTTGAAGGAAGCTGGTTACTACTTCCTAACACCTGATGAAACTTACCGTGTTCTTACTAAAAACGAAGTTCCATC
CGAAAAAATAGTCTGGTTGACTTTTGACGATAGCATGATTGATTTTTATCATGTAGCTTATCCAATTCTAAAAAAATACAA
TGCCAAGGCTACAAATAATGTCATCACTGGTTTAACAGAAAAAGCAAGTGTGGCTAATTTGACAGTCCCTCAAATGA
AAGAGATGAAAGAACATGGCATGTCCTTCCAAGACCATACTGTTAATCATCCTGACTTATCACAACAAGATTCTGCCACAC
AAGAACTTGAAATGAAAGATTCTATGGTTTACCTTAACCAAGAACTCGATCAAAAGACGATAGCTATTGCCTATCCGGCC
GGTCGTTATAATGAGACAACACTTGATATCGCAAAACAGTTAGACTATCAATTAGGTTTAACAACAAATGAAGGACTAGC
CAGTGCTGATGATGGTTTACTTTC

7) T2-53, I26 & T2-5
CAAAACTTTTACCTTCTCAAAACAGAACTGAGACTGTCAATAAGTCTAACAAGGTTTCAAAAAATACCACTAATCAGGCCA
AAAAAAGGCAATCTCTTTCCAATAATTCTCAAACTTGGATCAAAAAAACTAGTCCTGTTAAAGTTCCTATTTTAATGTACC
ACGCTATCCATGTTATGGCTCCTGAGGAAGCAGCAAATGCCAATTTAATTGTTGCACCCGACCTTTTCGAAATTCAAATC
AAAGCCTTGAAGGAAGCTGGTTACTACTTCCTAACACCTGAAGAAACTTACCGTGTTCTTACTAAAAACGAAGTTCCATC
CGAAAAAATAGTCTGGTTGACTTTTGACGATAGCATGATTGATTTTTATCATGTAGCTTATCCAATTCTAAAAAAATACAA
TGCCAAGGCTACAAATAATGTCATCACTGGTTTAACAGAAAAAGCAAGTGTGGCTAATTTGACAGTCCCTCAAATGA
AAGAGATGAAAGAACATGGCATGTCCTTCCAAGACCATACTGTTAATCATCCTGACTTATCACAACAAGATTCTGCCACAC
AAGAACTTGAAATGAAAGATTCTATGGTTTACCTTAACCAAGAACTCGATCAAAAGACGATAGCTATTGCCTATCCGGCC
GGTCGTTATAATGAGACAACACTTGATATCGCAAAACAGTTAGACTATCAATTAGGTTTGACAACAAATGAAGGACTAGC
CAGTGCTGATGATGGTTTACTTTC

8) T2-73
CAAAACTTTTACCTTCTCAAAACAGAACTGAGACTGTCAATAAGTCTAACAAGGTTTCAAAAAATACCACTAATCAGGCCA
AAAAAAGGCAATCTCTTTCCAATGATTCTCAAACTTGGATCAAAAAAACTAGTCCTGTTAAAGTTCCTATTTTAATGTACC
ACGCTATCCATGTTATGGCTCCTGAGGAAGCAGCAAATGCCAATTTAATTGTTGCACCCGACCTTTTCGAAAGTCAAATC
AAAGCCTTGAAGGAAGCTGGTTACTACTTCCTAACACCTGAAGAAACTTACCGTGTTCTTACTAAAAACGAAGTTCCATC
TGAAAAAATAGTCTGGTTGACTTTTGACGATAGCATGATTGATTTTTATCATGTAGCTTATCCAATTCTAAAAAAATACAA
TGCCAAGGCTACAAATAATGTCATCACTGGTTTAACAGAAAAAGCAAGTGTGGCTAATTTGACAGTCCCTCAAATGA
AAGAGATGAAAGAACATGGCATGTCCTTCCAAGACCATACTGTTAATCATCCTGACTTATCACAACAAGATTCTGCCACAC
AAGAACTTGAAATGAAAGATTCTATGGTTTACCTTAACCAAGAACTCGATCAAAAGACGATAGCTATTGCCTATCCGGCC
GGTCGTTATAATGAGACAACACTTAATATCGCAAAACAGTTAGACTATCAATTAGGTTTAACAACAAATGAAGGACTAGC
CAGTGCTGATGATGGTTTACTTTC

9) I23
CAAAACTTTTACCTTCTCAAAACAGAACTGAGACTGTCAATAAGTCTAACAAGGTTTCAAAAAATACCACTAATCAGGCCA
AAAAAAGGCAATCTCTTTCCAATGATTCTCAAACTTGGATCAAAAAAACTAGTCCTGTTAAAGTTCCTATTTTAATGTACC
ACGCTATCCATGTTATGGCTCCTGAGGAAGCAGCAAATGCCAATTTAATTGTTGCACCCGACCTTTTCGAAAGTCAAATC
AAAGCCTTGAAGGAAGCTGGTTACTACTTCCTAACACCTGAAGAAACTTACCGTGTTCTTACTAAAAACGAAGTTCCATC
CGAAAAAATAGTCTGGTTGACTTTTGACGATAGCATGATTGATTTTTATCATGTAGCTTATCCAATTCTAAAAAAATACAA
TGCCAAGGCTACAAATAATGTCATCACTGGTTTAACAGAAAAAGCAAGTGTGGCTAATTTGACAGTCCCTCAAATGA
AAGAGATGAAAGAACATGGCATGTCCTTCCAAGACCATACGGTTAATCATCCTGACTTATCACAACAAGATTCTGCCACA
CAAGAACTTGAAATGAAAGATTCTATGGTTTACCTTAACCAAGAACTCGATCAAAAGACGATAGCTATTGCCTATCCGGC
CGGTCGTTATAATGAGACAACACTTGATATCGCAAAACAGTTAGACTATCAATTAGGTTTAACAACAAATGAAGGACTAG
CCAGTGCTGATGATGGTTTACTTTC

10) 20569 & 1.93
CAAAACTTTTACCTTCTCAAAACAGAACTGAGACTGTCAATAAGTCTAACAAGGTTTCAAAAAATACCACTAATCAGGCCA
AAAAAAGGCAATCTCTTTCCAATGATTCTCAAACTTGGATCAAAAAAACTAGTCCTGTTAAAGTTCCTATTTTAATGTACC
ACGCTATCCATGTTATGGCTCCTGAGGAAGCAGCAAATGCCAATTTAATTGTTGCACCCGACCTTTTCGAAAGTCAAATC
AAAGCCTTGAAGGAAGCTGGTTACTACTTCCTAACACCTGAAGAAACTTACCGTGTTCTTACTAAAAACGAAGTTCCATC
CGAAAAAATAGTCTGGTTGACTTTTGACGATAGCATGATTGATTTTTATCATGTAGCTTATCCAATTCTAAAAAAATACAA
TGCCAAGGCTACAAATAATGTCATCACTGGTTTAACAGAAAAAGCAAGTGTGGCTAATTTGACAGTCCCTCAAATGA
AAGAGATGAAAGAACATGGCATGTCCTTCCAAGACCATACTGTTAATCATCCTGACTTATCACAACAAGATTCTGCCACAC
AAGAACTTGAAATGAAAGATTCTATGGTTTACCTTAACCAAGAACTCGATCAAAAGGACGATAGCTATTGCCTATCCGGCC
GGTCGTTATAATGAGACAACACTTGATATCGCAAAACAGTTAGACTATCAATTAGGTTTAACAACAAATGAAGGACTAGC
CAGTGCTGATGATGGTTTACTTTC

11) T1-36
CAAAACTTTTACCTTCTCAAAACAGAACTGAGACTGTCAATAAGTCTAACAAGGTTTCAAAAAATACCACTAATCAGGCCA
AAAAAAGGCAATCTCTTTCCAATGATTCTCAAACTTGGATCAAAAAAACTAGTCCTGTTAAAGTTCCTATTTTAATGTACC
ATGCTATCCATGTTATGGCTCCTGAGGAAGCAGCAAATGCCAATTTAATTGTTGCACCCGACCTTTTCGAAAGTCAAATC
AAAGCCTTGAAGGAAGCTGGTTACTACTTCCTAACACCTGAAGAAACTTACCGTGTTCTTACTAAAAACGAAGTTCCATC
TGAAAAAATAGTCTGGTTGACTTTTGACGATAGCATGATTGATTTTTATCATGTAGCTTATCCAATTCTAAAAAAATACAA
TGCCAAGGCTACAAATAATGTCATCACTGGTTTAACAGAAAAAGCAAGTGTGGCTAATTTGACAGTCCCTCAAATGA
AAGAGATGAAAGAACATGGCATGTCCTTCCAAGACCATACTGTTAATCATCCTGACTTATCACAACAAGATTCTGCCACAC
AAGAACTTGAAATGAAAGATTCTATGGTTTACCTTAACCAAGAACTCGATCAAAAGACGATAGCTATTGCCTATCCGGCC
GGTCGTTATAATGAGACAACACTTGATATCGCAAAACAGTTAGACTATCAATTAGGTTTAACAACAAATGAAGGACTAGC
CAGTGCTGATGATGGTTTACTTTC

12) T1-22
CAAAACTTTTACCTTCTCAAAACAGAACTGAGACTGTCAATAAGTCTAACAAGGTTTCAAAAAATACCACTAATCAGGCCA
AAAAAAGGCAATCTCTTTCCAATAATTCTCAAACTTGGATCAAAAAAACTAGTCCTGTTAAAGTTCCTATTTTAATGTACC
ACGCTATCCATGTTATGGCTCCTGAGGAAGCAGCAAATGCCAATTTAATTGTTGCACCCGACCTTTTCGAAAGTCAAATC
AAAGCCTTGAAGGAAGCTGGTTACTACTTCCTAACACCTGAAGAAACTTACCGTGTTCTTACTAAAAACGAAGTTCCATC
CGAAAAAATAGTCTGGTTGACTTTTGACGATAGCATGATTGATTTTTATCATGTAGCTTATCCAATTCTAAAAAAATACAA
TGCCAAGGCTACAAATAATGTCATCACTGGTTTAACAGAAAAAGCAAGTGTGGCTAATTTGACAGTCCCTCAAATGAAAG
AGATGAAAGAACATGGCATGTCCTTCCAAGACCATACTGTTAATCATCCTGACTTATCACAACAAGATTCTGCCACACAAG
AACTTGAAATGAAAGATTCTATGGTTTACCTTAACCAAGAACTCGATCAAAAGACGATAGCTATTGCCTATCCGGCCGGT
CGTTATAATGAGACAACACTTGATATCGCAAAACAGTTAGACTATCAATTAGGTTTGACAACAAATGAAGGACTAGCCAG
TGCTGATGATGGTTTACTTTC

13) I38 & I40
CAAAACTTTTACCTTCTCAAAACAGAACTGAGACTGTCAATAAGTCTAACAAGGTTTCAAAAAATACCACTAATCAGGCCA
AAAAAAGGCAATCTCTTTCCAATAATTCTCAAACTTGGATCAAAAAAACTAGTCCTGTTAAAGTTCCTATTTTAATGTACC
ACGCTATCCATGTTATGGCTCCTGAGGAAGCAGCAAATGCCAATTTAATTGTTGCACCCGACCTTTTCGAAAGTCAAATC
AAAGCCTTGAAGGAAGCTGGTTACTACTTCCTAACACCTGAAGAAACTTACCGTGTTCTTACTAAAAACGAAGTTCCATC

CGAAAAAATAGTCTGGTTGACTTTTGACGATAGCATGATTGATTTTTATCATGTAGCTTATCCAATTCTAAAAAAATACAA
TGCCAAGGCTACAAATAATGTCATCACTGGTTTAACAGAAAAAGCAAGTGTGGCTAATTTGACAGTCCCTCAAATGAAAG
AGATGAAAGAACATGGCATGTCCTTCCAAGACCATACTGTTAATCATCCTGACTTATCACAACAAGATTCTGCCACACAAG
AACTTGAAATGAAAGATTCTATGGTTTACCTTAACCAAGAACTCGATCAAAAGACGATAGCTATTGCCTATCCGGCCGGT
CGTTATAATGAGACAACACTTGATATCGCAAAACAGTTAGACTATCAATTAGGTTTAACAACAAATGAAGGACTAGCCAG
TGCTGATGATGGTTTACTTTC

14) I14
CAAAACTTTTACCTTCTCAAAACAGAACTGAGACTGTCAATAAGTCTAACAAGGTTTCAAAAAATACCACTAATCAGGCCA
AAAAAAGGCAATCTCTTTCCAATGATTCTCAAACTTGGATCAAAAAAACTAGTCCTGTTAAAGTTCCTATTTTAATGTACC
ACGCTATCCATGTTATGGCTCCTGAGGAAGCAGCAAATGCCAATTTAATTGTTGCACCCGACCTTTTCGAAAGTCAAATC
AAAGCCTTGAAGGAAGCTGGTTACTACTTCCTAACACCTGAAGAAACTTACCGTGTTCTTACTAAAAACGAAGTTCCATC
TGAAAAAATAGTCTGGTTGACTTTTGACGATAGCATGATTGATTTTTATCATGTAGCTTATCCAATTCTAAAAAAATACAA
TGCCAAGGCTACAAATAATGTCATCACTGGTTTAACAGAAAAAGCAAGTGTGGCTAATTTGACAGTCCCTCAAATGAAAG
AGATGAAAGAACATGGCATGTCCTTCCAAGACCATACTGTTAATCATCCTGACTTATCACAACAAGATTCTGCCACACAAG
AACTTGAAATGAAAGATTCTATGGTTTACCTTAACCAAGAACTCGATCAAAAGACGATAGCTATTGCCTATCCGGCCGGT
CGTTATAATGAGACAACACTTGATATCGCAAAACAGTTAGACTATCAATTAGGTTTAACAACAAATGAAGGACTAGCCAG
TGCTGATGATGGTTTACTTTC

15) I2 & I3
CAAAACTTTTACCTTCTCAAAACAGAACTGAGACTGTCAATAAGTCTAACAAGGTTTCAAAAAATACCACTAATCAGGCCA
AAAAAAGGCAATCTCTTTCCAATGATTCTCAAACTTGGATCAAAAAAACTAGTCCTGTTAAAGTTCCTATTTTAATGTACC
ACGCTATCCATGTTATGGCTCCTGAGGAAGCAGCAAATGCCAATTTAATTGTTGCACCCGACCTTTTCGAAAGTCAAATC
AAAGCCTTGAAGGAAGCTGGTTACTACTTCCTAACACCTGAAGAAACTTACCGTGTTCTTACTAAAAACGAAGTTCCATC
CGAAAAAATAGTCTGGTTGACTTTTGACGATAGCATGATTGATTTTTATCATGTAGCTTATCCAATTCTAAAAAAATACAA
TGCCAAGGCTACAAATAATGTCATCACTGGTTTAACAGAAAAAGCAAGTGTGGCTAATTTGACAGTCCCTCAAATGAAAG
AGATGAAAGAACATGGCATGTCCTTCCAAGACCATACTGTTAATCATCCTGACTTATCACAACAAGATTCTGCCACACAAG
AACTTGAAATGAAAGATTCTATGGTTTACCTTAACCAAGAACTCGATCAAAAGACGATAGCTATTGCCTATCCGGCCGGT
CGTTATAATGAGACAACACTTGATATCGCAAAACAGTTAGACTATCAATTAGGTTTAACAACAAATGAAGGACTAGCCAG
TGCTGATGATGGTTTACTTTC

SUB 1487 (*icaC* homologue) sequences

1) T2-73, I10, I26, I38, I40, T1-22, T1-36, T1-43, T1-60, T2-1, T2-5, T2-10, T2-11, T2-36, T2-53 & T2-67
TGGATATGTTGGTATTCAATCACGCTATAAATATTCGAAATTAGCCAATATTTGGCTTCAAGTTTTTTTCTATACCTTTAC
GATTACATCTTTATTTGCACTTTCAGGATTTCCAATAACTGCCTTGAATTGGCGTCATGCCTTTTTTCCAATAGTCAGTG
GTGAATATTGGTACATGACAGCATATTTTGGTCTACTCATTTTTATGCCGATGATTAATGCTGGTATTAATGCATTAACG
GATAGGCAATTAAAGCATTTGGTTATTTTGATGGCAGTTGTTTTTTCAATCTTACCTGCTATGATGAATAACCGGGTTGC
TGAGTTTTCACTGAGTAAAGGTTTTGAGATGACATGGCTAATGATTTTATATATTGTTGGAGCCTATCTTCATCGTTTGA
ATTTAAACCAATTTAGTGGAAAGAAATTATTAGCAACTTATTTTGGATGTATGGTTGTAACCTTTGCGATGAAATTTATG
GTTGGTAACATTTGGTATTGGTATGTGTCTCCAAGTTTGAGTTTAGGAGCTATTAGTTTATTCATACTATTTGCCAAAAT
GAAAATTAAAAAAGGTTCTTTATTGTTTAAATGGATTGTTTTAATTTCGCCAACGACTTTAGGTGTCTACTTGGCTCATTT
ACATC

2) 1.93
TGGATATGTTGGTATTCAATCACGCTATAAATATTCGAAATTAGCCAATATTTGGCTTCGAGTTTTTTTCTATACCTTTAC
GATTACATCTTTATTTGCACTTTCAGGATTTCCAATAACTACCTTGAATTGGCGTCATGCCTTTTTTCCAATAGTCAGTGG
TGAATATTGGTACATGACAGCATATTTTGGTCTACTCATTTTTATGCCGATGATTAATGCTGGTATTAATGCATTAACGG
ATAGGCAATTAAAGCATTTGGTTATTTTGATGGCAGTTGTTTTTTCAATCTTACCTGCTATGATGAATAACCGTGTTGCT
GAGTTTTCACTGAGTAAAGGTTTTGAGATGACATGGCTAATGATTTTATATATTGTTGGAGCCTATCTTCATCGTTTGAA
TTTAAACCAATTTAGTGGAAAGAAATTATTAGCAACTTATTTTGGATGTATGGTTGTAACCTATGCGATGAAATTTATGG
TTGGTAACATTTGGTATTGGTATGTGTCTCCAAGTTTGAGTTTAGGAGCTATTAGTTTATTCATACTATTTGCCAAAATG
AAAATTAAAAAAGGTTCTTTATTGTTTAAATGGATTGTTTTAATTTCGCCAACGACTTTAGGTGTCTACTTGGCTCATTTA
CATC

3) 0140J
TGGATATGTTGGTATTCAATCACGCTATAAATATTCGAAATTAGCCAATATTTGGCTTCAAGTTTTTTTCTATACCTTTAC
GATTACATCTTTATTTGCACTTTCAGGATTTCCAATAACTGCCTTGAATTGGCGTCATGCCTTTTTTCCAATAGTCAGTG
GTGAATATTGGTACATGACAGCATATTTTGGTCTACTCATTTTTATGCCGATGATTAATGCTGGTATTAATGCATTAACG
GATAGGCAATTAAAGCATTTGGTTATTTTGATGGCAGTTGTTTTTTCAATCTTACCTGCTATGATGAATAACCGGGTTGC
TGAGTTTTCACTGAGTAAAGGTTTTGAGATGACATGGCTAATGATTTTATATATTGTTGGAGCCTATCTTCATCGTTTGA
ATTTAAACCAATTTAGTGGAAAGAAATTATTAGCAACTTATTTTGGATGTATGGTTGTAACCTTTGTGATGAAATTTATG
GTTGGTAACATTTGGTATTGGTATGTGTCTCCAAGTTTGAGTTTAGGAGCTATTAGTTTATTCATACTATTTGCCAAAAT
GAAAATTAAAAAAGGTTCTTTATTGTTTAAATGGATTGTTTTAATTTCGCCAACGACTTTAGGTGTCTACTTGGCTCATTT
ACATC

4) 20569 & T1-20
TGGATATGTTGGTATTCAATCACGCTATAAATATTCGAAATTAGCCAATATTTGGCTTCAAGTTTTTTTCTATACCTTTAC
GATTACATCTTTATTTGCACTTTCAGGATTTCCAATAACTGCCTTGAATTGGCGTCATGCCTTTTTTCCAATAGTCAGTG
GTGAATATTGGTACATGACAGCATATTTTGGTCTACTCATTTTTATGCCGATGATTAATGCTGGTATTAATGCATTAACG
GATAAGCAATTAAAGCATTTGGTTATTTTGATGGCAGTTGTTTTTTCAATCTTACCTGCTATGATGAATAACCGGGTTGC
TGAGTTTTCACTGAGTAAAGGTTTTGAGATGACATGGCTAATGATTTTATATATTGTTGGAGCCTATCTTCATCGTTTGA
ATTTAAACCAATTTAGTGGAAAGAAATTATTAGCAACTTATTTTGGATGTATGGTTGTAACCTTTGCGATGAAATTTATG
GTTGGTAACATTTGGTATTGGTATGTGTCTCCAAGTTTGAGTTTAGGAGCTATTAGTTTATTCATACTATTTGCCAAAAT
GAAAATTAAAAAAGGTTCTTTATTGTTTAAATGGATAGTTTTAATTTCGCCAACGACTTTAGGTGTCTACTTGGCTCATTT
ACATC

5) I2
TGGATATGTTGGTATTCAATCACGCTATAAATATTCGAAATTAGCCAATATTTGGCTTCAAGTTTTTTTCTATACCTTTAC
GATTACATCTTTATTTGCACTTTCAGGATTTCCAATAACTGCCTTGAATTGGCGTCATGCCTTTTTTCCAATAGTCAGTG
GTGAATATTGGTACATGACAGCATATTTTGGTCTACTCATTTTTATGCCGATGATTAATGCTGGTATTAATGCATTAACG
GATAGGCAATTAAAGCATTTGGTTATTTTGATGGCAGTTGTTTTTTCATTCTTACCTGCTATGATGAATAACCGGGTTGC
TGAGTTTTCACTGAGTAAAGGTTTTGAGATGACATGGCTAATGATTTTATATATTGTTGGAGCCTATCTTCATCGTTTGA
ATTTAAACCAATTTAGTGGAAAGAAATTATTAGCAACTTATTTTGGATGTATGGTTGTAACCTATGCGATGAAATTTATG
GTTGGTAACATTTGGTATTGGTATGTGTCTCCAAGTTTGAGTTTAGGAGCTATTAGTTTATTCATACTATTTGCCAAAAT
GAAAATTAAAAAAGGTTCTTTATTGTTTAAATGGATTGTTTTAATTTCGCCAACGACTTTAGGTGTCTACTTGGCTCATTT
ACATC

6) I3
TGGATATGTTGGCATTCAATCACGCTATAAATATTCGAAATTAGCCAATATTTGGCTTCAAGTTTTTTTCTATACCTTTAC
GATTACATCTTTATTTGCACTTTCAGGATTTCCAATAACTGCCTTGAATTGGCGTCATGCCTTTTTTCCAATAGTCAGTG
GTGAATATTGGTACATGACAGCATATTTTGGTCTACTCATTTTTATGCCGATGATTAATGCTGGTATTAATGCATTAACG
GATAGGCAATTAAAGCATTTGGTTATTTTGATGGCAGTTGTTTTTTCAATCTTACCTGCTATGATGAATAACCGGGTTGC
TGAGTTTTCACTGAGTAAAGGTTTTGAGATGACATGGCTAATGATTTTATATATTGTTGGAGCCTATCTTCATCGTTTGA
ATTTAAACCAATTTAGTGGAAAGAAATTATTAGCAACTTATTTTGGATGTATGGTTGTAACCTATGCGATGAAATTTATG
GTTGGTAACATTTGGTATTGGTATGTGTCTCCAAGTTTGAGTTTAGGAGCTATTAGTTTATTCATACTATTTGCCAAAAT
GAAAATTAAAAAAGGTTCTTTATTGTTTAAATGGATTGTTTTAATTTCGCCAACGACTTTAGGTGTCTACTTGGCTCATTT
ACATC

7) I6
TGGATATGTTGGTATTCAATCACGCTATAAATATTCGAAATTAGCCAATATTTGGCTTCAAGTTTTTTTCTATACCTTTAC
GATTACATCTTTATTTGCACTTTCAGGATTTCCAATAACTGCCTTGAATTGGCGTCATGCCTTTTTTCCAATAGTCAGTG
GTGAATATTGGTACATGACAGCATATTTTGGTCTACTCATTTTTATGCCGATGATTAATGCTGGTATTAATGCATTAACG
GATAGGCAATTAAAGCATTTGGTTATTTTGATGGCAGTTGTTTTTTCAATCTTACCTGCTATGATGAATAACCGGGTTGC
TGAGTTTTCACTGAGTAAAGGTTTTGAGATGACATGGCTAATGATTTTATATATTGTTGGAGCCTATCTTCATCGTTTGA
ATTTAAACCAATTTAGTGGAAAGAAATTATTAGCAACTTATTTTGGATGTATGGTTGTAACCTATGCGATGAAATTTATG
GTTGGTAACATTTGGTATTGGTATGTGTCTCCAAGTTTGAGTTTAGGAGCTATTAGTTTATTCATACTATTTGCCAAAAT
GAAAATTAAAAAAGGTTCTTTATTATTTAAATGGATTGTTTTAATTTCGCCAACGACTTTAGGTGTCTACTTGGCTCATTT
ACATC

8) I14
TGGATATGTTGGTATTCAATCACGCTATAAATATTCGAAATTAGCCAATATTTGGCTTCAAGTTTTTTTCTATACCTTTAC
GATTACATCTTTATTTGCACTTTCAGGATTTCCAATAACTGCCTTGAATTGGCGTCATGCCTTTTTTCCAATAGTCAGTG
GTGAATATTGGTACATGACAGCATATTTTGGTCTACTCATTTTTATGCCGATGATTAATGCTGGTATTAATGCATTAACG
GATAGGCAATTAAAGCATTTGGTTATTTTGATGGCAGTTGTTTTTTCAATCTTACCTGCTATGATGAATAACCGGGTTGC
TGAGTTTTCACTGAGTAAAGGTTTTGAGATGACATGGCTAATGATTTTATATATTGTTGGAGCCTATCTTCATCGTTTGA
ATTTAAACCAATTTAGTGGAAAGAAATTATTAGCAACTTATTTTGGATGTATGGTTGTAACCTATGCGATGAAATTTATG
GTTGGTAACATTTGGTATTGGTATGTGTCTCCGAGTTTGAGTTTAGGAGCTATTAGTTTATTCATACTATTTGCCAAAAT
GAAAATTAAAAAAGGGTCTTTATTGTTCAAATGGATTGTTTTAATTTCGCCAACGACATTAGGTGTCTACTTGGCTCATT
TACATC

9) I23
TGGATATGTTGGTATTCAATCACGCTATAAATATTCGAAATTAGCCAATATTTGGCTTCAAGTTTTTTTCTATACCTTTAC
GATTACATCTTTATTTGCACTTTCAGGATTTCCAATAACTGCCTTGAATTGGCGTCATGCCTTTTTTCCAATAGTCAGTG
GTGAATATTGGTACATGACAGCATATTTTGGTCTACTCATTTTTATGCCGATGATTAATGCTGGTATTAATGCATTAACG
GATAGGCAATTAAAGCATTTGGTTATTTTGATGGCAGTTGTTTTTTCAATCTTACCTGCTATGATGAATAACCGTGTTGC
TGAGTTTTCACTGAGTAAAGGTTTTGAGATGACATGGCTAATGATTTTATATATTGTTGGAGCCTATCTTCATCGTTTGA
ATTTAAACCAATTTAGTGGAAAGAAATTATTAGCAACTTATTTTGGATGTATGGTTGTAACCTATGCGATGAAATTTATG
GTTGGTAACATTTGGTATTGGTATGTGTCTCCAAGTTTGAGTTTAGGAGCTATTAGTTTATTCATACTATTTGCCAAAAT
GAAAATTAAAAAAGGTTCTTTATTGTTTAAATGGATTGTTTTAATTTCGCCAACGACTTTAGGTGTCTACTTGGCTCATTT
ACATC

10) I34
TGGATATGTTGGTATTCAATCACGCTATAAATATTCGAAATTAGCCAATATTTGGCTTCAAGTTTTTTTCTATACCTTTAC
GATTACATCTTTATTTGCACTTTCAGGATTTCCAATAACTGCCTTGAATTGGCGTCATGCCTTTTTTCCAATAGTCAGTG
GTGAATATTGGTACATGACAGCATATTTTGGTCTACTCATTTTTATGCCGATGATTAATGCTGGTATTAATGCATTAACG
GATAGGCAATTAAAGCATTTGGTTATTTTGATGGCAGTTGTTTTTTCAATCTTACCTGCTATGATGAATAACCGGGTTGC
TGAGTTTTCACTGAGTAAAGGTTTTGAGATGACATGGCTAATGATTTTATATATTGTTGGAGCCTATCTTCATCGTTTGA
ATTTAAACCAATTTAGTGGAAAGAAATTATTAGCAACTTATTTTGGATGTATGGTTGTAACCTATGCGATGAAATTTATG
GTTGGTAACATTTGGTATTGGTATGTGTCTCCAAGTTTGAGTTTAGGAGCTATTAGTTTATTCATACTATTTGCCAAAAT
GAAAATTAAAAAAGGTTCTTTATTGTTTAAATGGATTGTTTTAATTTCGCCAACGACTTTAGGTGTCTACTTGGCTCATTT
ACATC

SUB 0701 (*icaD* homologue) sequences

1) 0140J, T2-1, T2-5, T2-36
CTAAAATTTAAAATGAGAAGTGAACCCCTTCTGATAACTGATTTTGCATGGTTAAAAGATTTAAAACTAGTTTTTAGCTTT
TTGGATTTAAAATATATTATTTATTCTCTAATTTTGATAGTCTTACCTATCCTTGTTTTCTTTCTATTTCGAAAAAGATTCT
TTAATATTAAGGTATTCAAAAATATTTTCTTTAGAGTAGGAGTTCTATTTAGCATTTTATTGACTTTTTACACTTTAACAC
TGATTTTCAAAAATGAAATTAAAGGTAAAATTCAAGATAATATACCTGTAGTATCAAAATTAAATAATAAATTAGATATTG
CCTATATGGGACATTTAACCAATGCAAGATATAAATCAGTAGCTTATGTTTGGACAAAACAAATCAGTAAACCGATTATG
GAAAAGCCTGACAATTATTCAAAAAACGAAGTACAAAGAATTGTTAAAAAATACACCCGACGTGCAGCCGAAATAAATAG
TACGAGGGATAATAATTTATCGG

2) T1-36
CTAAAATTTAAAATGAGAAGTGAACCCCTTCTGATAACTGATTTTGCATGGTTAAAAGATTTAAAACTAGTTTTTAGCTTT
TTGGATTTAAAATATATTATTTATTCTCTAATTTTGATAGTCTTACCTATCCTTGTTTTCTTTCTATTTCGAAAAAGATTCT
TTAATATTAAGGTATTCAAAAATATTTTCTTTAGAGTAGGAGTTCTATTTAGCATTTTATTGACTTTTTACACTTTAACAC
TGATTTTCAAAAATGAAATTAAAGGTAAAATTCAAGATAATATACCTGTAGTATCAAAATTAAATAATAAATTAGATATTG
CCTATATGGGACATTTAACCAATGCAAGATATAAATCAGTAGCTTATGTTTGGACAAAACAAATCAGTAAACCGATTATG
GAAAAGCCTGACAATTATTCAAAAAACGAAGTACAAAGAATTGTTAAAAAATATACCCGACGCGCAGCCGAAATAAATAG
TACGAGGGATAATAATTTATCGG

luxS (*luxS* homologue) sequences

1) T2-73, 1.93, 0140J, I26, I38, T1-36, T1-43, T2-5, T2-10, T2-11, T2-36 & T2-53
ATTTTTAGAGAATCCTCAATTTTTCTTCTGTTTTATGTTAAGATGGATGGAGAAGAAAAAAGGAGATTAACTATGACTAA
AGAAGTAATTGTTGAAAGTTTTGAATTAGACCATACCATTGTTAAAGCCCCTTATGTACGATTAATTTCAGAAGAGTTTG
GTCCTAAGGGAGATGTTATCACTAATTTTGATGTTCGCTTGGTTCAACCAAACCAAAATTCCATTGAAACAGCTGGATTA
CACACCATCGAACATCTCTTAGCAAAACTGATTCGCCAACGTATTGATGGCATGATTGATTGTTCTCCCTTTGGTTGCCG
AACAGGTTTCCATTTAATCATGTGGGGAGAGCATAGTTCAACAGAAATTGCAAAGGTCATCAAATCAAGCTTAGAAGAAA
TTGCTGACGGCATTTCCTGGGAAGATGTTCCAGGTACTACCATTGAATCTTGTGGTAATTATAAGGACCATAGCTTATTT
GCTGCAAAAGAATGGGCAAAACTAATC

2) 20569
ATTTTTAGAGAATCCTCAATTTTTCTTCTGTTTTATGTTAAGATGGATGGAGAAGAAAAAAGGAGATTAACTATGACTAA
AGAAGTAATTGTTGAAAGTTTTGAATTAGACCATACCATTGTTAAAGCCCCTTATGTACGATTAATTTCAGAAGAGTTTG
GTCCTAAGGGAGATATTATCACTAATTTTGATGTTCGCTTGGTTCAACCAAACCAAAATTCCATTGAAACAGCTGGATTA
CACACCATCGAACATCTCTTAGCAAAACTGATTCGCCAACGTATTGATGGCATGATTGATTGTTCTCCCTTTGGTTGCCG
AACAGGTTTCCATTTAATCATGTGGGGAAAGCATAGTTCAACAGAAATTGCAAAGGTCATCAAATCAAGCTTAGAAGAAA
TTGCTAACGGCATTTCCTGGGAAGATGTTCCAGGTACTACCATTGAATCTTGTGGTAATTATAAGGACCATAGCTTATTT
GCTGCAAAAGAATGGGCAAAACTAATC

3) I2 & I23
ATTTTTAGAGAATCCTCAATTTTTCTTCTGTTTTATGTTAAGATGGATGGAGAAGAAAAAAGGAGATTAACTATGACTAA
AGAAGTAATTGTTGAAAGTTTTGAATTAGACCATACCATTGTTAAAGCCCCTTATGTACGATTAATTTCAGAAGAGTTTG
GTCCTAAGGGAGATGTTATCACTAATTTTGATGTACGCTTGGTTCAACCAAACCAAAATTCCATTGAAACAGCTGGATTA
CACACCATTGAACATCTCTTAGCAAAACTGATTCGCCAACGTATTGATGGCATGATTGATTGTTCTCCCTTTGGTTGCCG
AACAGGTTTCCATTTAATCATGTGGGGAGAGCATAGTTCAACAGAAATTGCAAAGGTCATCAAATCAAGCTTAGAAGAAA
TTGCTGACGGCATTTCCTGGGAAGATGTTCCAGGTACTACCATTGAATCTTGTGGTAATTATAAGGACCATAGCTTATTT
GCTGCAAAAGAATGGGCAAAACTAATC

4) I3

ATTTTTAGAGAATCCTCAATTTTTCTTCTGTTTTATGTTAAGATGGATGGAGAAGAAAAAAGGAGATTAACTATGACTAA
AGAAGTAATTGTTGAAAGTTTTGAATTAGACCATACCATTGTTAAAGCCCCTTATGTACGATTAATTTCAGAAGAGTTTG
GTCCTAAGGGAGATGTTATCACTAATTTTGATGTACGCTTGGTTCAACCAAACCAAAATTCCATTGAAACAGCTGGATTA
CACACCATTGAACATCTCTTAGCAAAACTGATTCGCCAACGTATTGATGGCATGATTGATTGTTCTCCCTTTGGTTGCCG
AACAGGTTTCCATTTAATCATGTGGGGAAAGCATAGTTCAACAGAAATTGCAAAGGTCATCAAATCAAGCTTAGAAGAAA
TTGCTAACGGCATTTCCTGGGAAGATGTTCCAGGTACTACCATTGAATCTTGTGGTAATTATAAGGACCATAGCTTATTT
GCTGCAAAAGAATGGGCAAAACTAATC

5) I6, I10 & T2-67
ATTTTTAGAGAATCCTCAATTTTTCTTCTGTTTTATGTTAAGATGGATGGAGAAGAAAAAAGGAGATTAACTATGACTAA
AGAAGTAATTGTTGAAAGTTTTGAATTAGACCATACCATTGTTAAAGCCCCTTATGTACGATTAATTTCAGAAGAGTTTG
GTCCTAAGGGAGATGTTATCACTAATTTTGATGTTCGCTTGGTTCAACCAAACCAAAATTCCATTGAAACAGCTGGATTA
CACACCATCGAACATCTCTTAGCAAAACTGATTCGCCAACGTATTGATGGCATGATTGATTGTTCTCCCTTTGGTTGCCG
AACAGGTTTCCATTTAATCATGTGGGGAAAGCATAGTTCAACAGAAATTGCAAAGGTCATCAAATCAAGCTTAGAAGAAA
TTGCTGACGGCATTTCCTGGGAAGATGTTCCAGGTACTACCATTGAATCTTGTGGTAATTATAAGGACCATAGCTTATTT
GCTGCAAAAGAATGGGCAAAACTAATC

6) I14
ATTTTTAGAGAATCCTCAATTTTTCTTCTGTTTTATGTTAAGATGGATGGAGAAGAAAAAAGGAGATTAACTATGACTAA
AGAAGTAATTGTTGAAAGTTTTGAATTAGACCATACCATTGTTAAAGCCCCTTATGTACGATTAATTTCAGAAGAGTTTG
GTCCTAAGGGAGATGTTATTACTAATTTTGATGTTCGCTTGGTTCAACCAAACCAAAATTCCATTGAAACAGCTGGATTA
CACACCATCGAACATTTCTTAGCAAAACTGATTCGCCAACGTATTGATGGCATGATTGATTGTTCTCCCTTTGGTTGCCG
AACAGGTTTCCATTTAATCATGTGGGGAAAGCATAGTTCAACAGAAATTGCAAAGGTCATCAAATCAAGCTTAGAAGAAA
TTGCTGACGGCATTTCCTGGGAAGATGTTCCAGGTACTACCATTGAATCTTGTGGTAATTATAAGGACCATAGCTTATTT
GCTGCAAAAGAATGGGCAAAACTAATC

7) I34
ATTTTTAGAGAATCCTCAATTTTTCTTCTATTTTATGTTAAGATGGATGGAGAAGAAAAAAGGAGATTAACTATGACTAAA
GAAGTAATTGTTGAAAGTTTTGAATTAGACCATACCATTGTTAAAGCCCCTTATGTACGATTAATTTCAGAAGAGTTTGG
TCCTAAGGGAGATGTTATCACTAATTTTGATGTACGCTTGGTTCAACCAAACCAAAATTCCATTGAAACAGCTGGATTAC
ACACCATTGAACATCTCTTAGCAAAACTGATTCGCCAACGTATTGATGGCATGATTGATTGTTCTCCCTTTGGTTGCCGA
ACAGGTTTCCATTTAATCATGTGGGGAGAGCATAGTTCAACAGAAATTGCAAAGGTCATCAAATCAAGCTTAGAAGAAAT
TGCTGACGGCATTTCCTGGGAAGATGTTCCAGGTACTACCATTGAATCTTGTGGTAATTATAAGGACCATAGCTTATTTG
CTGCAAAAGAATGGGCAAAACTAATC

8) I40
ATTTTTAGAGAATCCTCAATTTTTCTTCTGTTTTATGTTAAGATGGATGGAGAAGAAAAAAGGAGATTAACTATGACTAA
AGAAGTAATTGTTGAAAGTTTTGAATTAGACCATACCATTGTTAAAGCCCCTTATGTACGATTAATTTCAGAAGAGTTTG
GTCCTAAGGGAGATGTTATCACTAATTTTGATGTTCGCTTGGTTCAACCAAACCAAGATTCCATTGAAACAGCTGGATTA
CACACCATCGAACATCTCTTAGCAAAACTGATTCGCCAACGTATTGATGGCATGATTGATTGTTCTCCCTTTGGTTGCCG
AACAGGTTTCCATTTAATCATGTGGGGAGAGCATAGTTCAACAGAAATTGCAAAGGTCATCAAATCAAGCTTAGAAGAAA
TTGCTGACGGCATTTCCTGGGAAGATGTTCCAGGTACTACCATTGAATCTTGTGGTAATTATAAGGACCATAGCTTATTT
GCTGCAAAAGAATGGGCAAAACTAATC

9) T1-20
ATTTTTAGAGAATCCTCAATTTTTCTTCTGTTTTATGTTAAGATGGATGGAGAAGAAAAAAGGAGATTAACTATGACTAA
AGAAGTAATTGTTGAAAGTTTTGAATTAGACCATACCATTGTTAAAGCCCCTTATGTACGATTAATTTCAGAAGAGTTTG
GTCCTAAGGGAGATGTTATCACTAATTTTGATGTACGCTTGGTTCAACCAAACCAAAATTCCATTGAAACAGCTGGATTA
CACACCATTGAACATCTCTTAGCAAAACTGATTCGCCAACGTATTGATGGCATGATTGATTGTTCTCCCTTTGGTTGCCG
AACAGGTTTCCATTTAATCATGTGGGGAAAGCATAGTTCAACAGAAATTGCAAAGGTCATCAAATCAAGCTTAGAAGAAA
TTGCTGACGGCATTTCCTGGGAAGATGTTCCAGGTACTACCATTGAATCTTGTGGTAATTATAAGGACCATAGCTTATTT
GCTGCAAAAGAATGGGCAAAACTAATC

10) T1-22
ATTTTTAGAGAATCCTCAATTTTTCTTCTGTTTTATGTTAAGATGGATGGAGAAGAAAAAAGGAGATTAACTATGACTAA
AGAAGTAATTGTTGAAAGTTTTGAATTAGACCATACCATTGTTAAAGCCCCTTATGTACGATTAATTTCTGAAGAGTTTG
GTCCTAAGGGAGATGTTATCACTAATTTTGATGTTCGCTTGGTTCAACCAAACCAAAATTCCATTGAAACAGCTGGATTA
CACACCATCGAACATCTCTTAGCAAAACTGATTCGCCAACGTATTGATGGCATGATTGATTGTTCTCCCTTTGGTTGCCG
AACAGGTTTCCATTTAATCATGTGGGGAAAGCATAGTTCAACAGAAATTGCAAAGGTCATCAAATCAAGCTTAGAAGAAA
TTGCTGACGGCATTTCCTGGGAAGATGTTCCAGGTACTACCATTGAATCTTGTGGTAATTATAAGGACCATAGCTTATTT
GCTGCAAAAGAATGGGCAAAACTAATC

11) T1-60
ATTTTTAGAGAATCCTCAATTTTTCTTCTGTTTTATGTTAAGATGGATGGAGAAGAAAAAAGGAGATTAACTATGACTAA
AGAAGTAATTGTTGAAAGTTTTGAATTAGACCATACCATTGTTAAAGCCCCTTATGTACGATTAATTTCTGAAGAGTTTG
GTCCTAAGGGAGATGTTATCACTAATTTTGATGTACGCTTGGTTCAACCAAACCAAAATTCCATTGAAACAGCTGGATTA

CACACCATTGAACATCTCTTAGCAAAACTGATTCGCCAACGTATTGATGGCATGATTGATTGTTCTCCCTTTGGTTGCCG
AACAGGTTTCCATTTAATCATGTGGGGAAAGCATAGTTCAACAGAAATTGCAAAGGTCATCAAATCAAGCTTAGAAGAAA
TTGCTGACGGCATTTCCTGGGAAGATGTTCCAGGTACTACCATTGAATCTTGTGGTAATTATAAGGACCATAGCTTATTT
GCTGCAAAAGAATGGGCAAAACTAATC

12) T2-1
ATTTTTAGAGAATCCTCAATTTTTCTTCTGTTTTATGTTAAGATGGATGGAGAAGAAAAAAGGAGATTAACTATGACTAA
AGAAGTAATTGTTGAAAGTTTTGAATTAGACCATACCATTGTTAAAGCCCCTTATGTACGATTAATTTCAGAAGAGTTTG
GTCCTAAGGGAGATGTTATCACTAATTTTGATGTACGCTTGGTTCAACCAAACCAAAATTCCATTGAAACAGCTGGATTA
CACACCATTGAACATCTCTTAGCAAAACTGATTCGCCAACGTATTGATGGCATGATTGATTGTTCTCCCTTTGGTTGTCG
AACAGGTTTCCATTTAATCATGTGGGGAAAGCATAGTTCAACAGAAATTGCAAAGGTCATCAAATCAAGCTTAGAAGAAA
TTGCTGACGGCATTTCCTGGGAAGATGTTCCAGGTACTACCATTGAATCTTGTGGTAATTATAAGGACCATAGCTTATTT
GCTGCAAAAGAATGGGCAAAACTAATC